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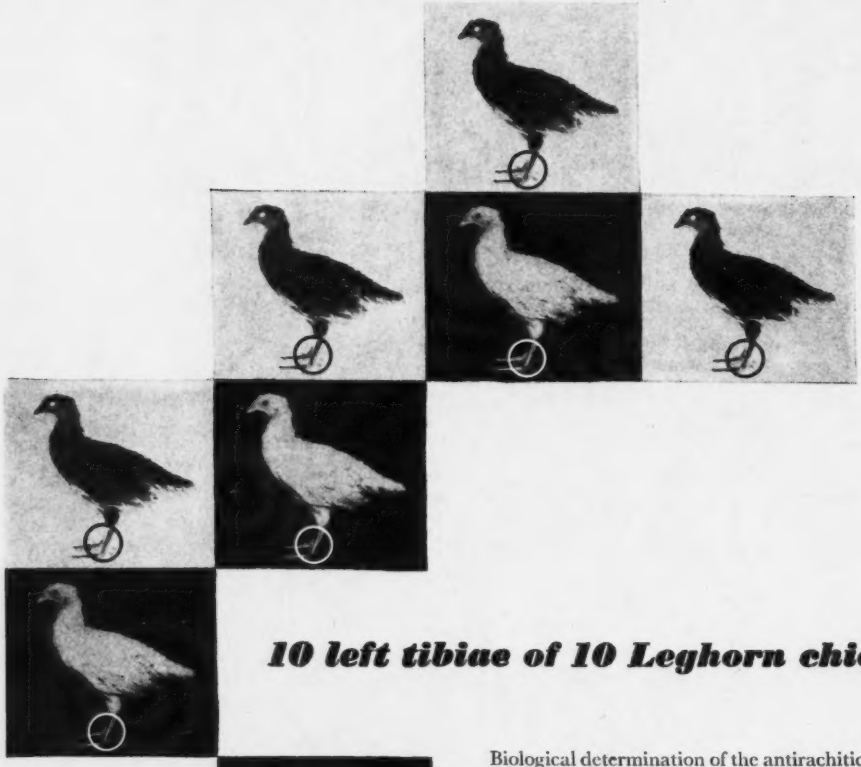

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ENDOCRINOLOGY

VOLUME 46 MAY, 1950 NUMBER 5

ON THE CONTROL OF FOLLICULAR DEVELOPMENT IN INTRASPLENIC OVARIAN GRAFTS BY MINUTE QUANTITIES OF OESTROGEN

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INTRODUCTION

THE CONTROL of ovarian endocrine function by oestrogen is known since almost twenty years (for references see Courrier 1945; Masson and Barsantini 1948; Robertson 1949). More recently it has been found in this Department that intrasplenic ovarian grafts in the guinea pig offer an excellent opportunity for a systematic study of the steroid control of follicular development dependent on hypophyseal gonadotrophins, the hypophysis being freed from the ovarian control since the steroids produced by the ovary itself are inactivated, when this device is used, in their passage through the liver. Hemorrhagic follicles (H.F.) and cystic follicles which are a characteristic feature of the intrasplenic graft in castrated females failed to appear when oestrogen absorbed from a subcutaneously implanted pellet of α-oestradiol was allowed to circulate in the body; production of corpora lutea was greatly enhanced. On the contrary, progesterone and desoxycorticosterone were shown to counteract luteinization (Lipschutz a. oth. 1948).[3]

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[1] Working for her Thesis of M.D. Univ. de Chile 1949.

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[3] Mention of previous important work related to the antiluteinizing action of progesterone has been unwillingly omitted in the paper of Lipschutz a.oth. See Dempsey a.oth. 1936; Dempsey 1937; Selye a.oth. 1936; Selye 1939 (many references). In the work of Dempsey luteinization was prevented by progesterone in guinea pigs; in the work of Selye in mice and rats. But subsequently results in the rat were contradictory (Selye and Friedman 1940). See also references in the book of Burrows (1949) and new work of Desclin (1949) and Kempf (1949).

Whereas these experimental results are clear cut, their interpretation as to the mechanisms involved and likewise their application to physiology are subject to many doubts. There is, first, the relevant question of the *quantity* of steroids which were allowed to circulate in the body. In the above experiments as much as 28 to 65 μg. of oestradiol were absorbed daily from the pellet during 45 days. This is probably a great amount compared to those quantities of oestrogen

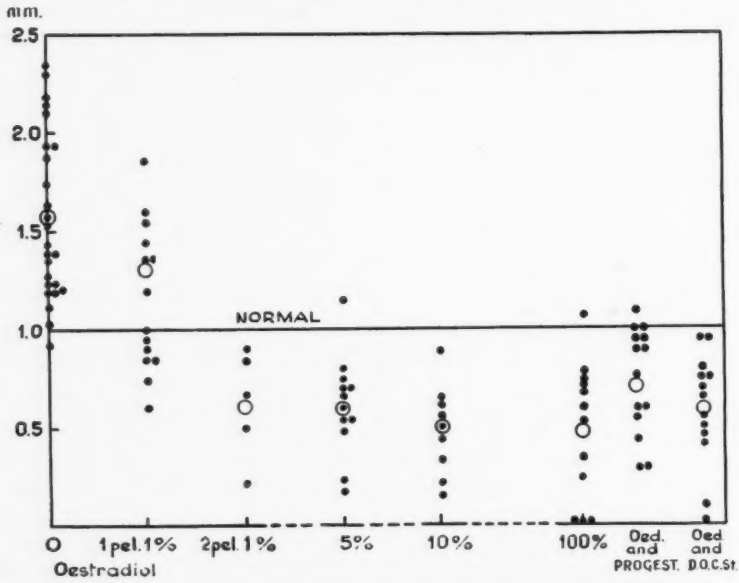

FIG. 1. Largest diameter of Graafian follicles. Each point corresponds to one animal. Circle—average of the group. Horizontal line at 1.0—largest diameter of Graafian follicles in ovary of normal adult guinea pig (average of 6 animals 1.0 ± 0.1, varying between 0.65 and 1.17). With the continuous action of oestradiol absorbed from two 1% pellets of oestradiol almost the same effect is produced as with pellets of pure oestradiol. The difference between the group with pure oestradiol pellets and the group with the simultaneous administration of progesterone sufficient to counteract luteinization is very small and scarcely significant; the average of the group with the simultaneous administration of desoxycorticosterone is very near to that with oestradiol alone.

which normally enter into the circulation in the guinea pig. In the present paper we shall deal with experiments in which oestrogen circulating in the body was greatly reduced; the subcutaneously implanted pellets from which oestrogen was absorbed contained only 1 to 10% of α-oestradiol mixed with cholesterol.

PROCEDURES

Procedures were similar to those in our former work. Pellets were implanted subcutaneously two weeks after castration and transplantation of the ovary. Weight of the pellets was of 20.2 to 34.4 mgr. The pellets with 1% of oestradiol contained a total of only 235 to 344 μg. of the hormone. Ne-

cropsy was made six weeks later. Two perpendicular diameters of the largest Graafian follicles were measured in representative sections projected on the screen.

EXPERIMENTAL RESULTS

Results are summarized in Table 1.

There is, first, the conclusive fact that with pellets containing 10 or 5% of oestradiol and even with two 1% pellets, H.F. were *always* suppressed. There was among 26 animals of groups I, II and III not a single one with H.F. (column 4 and 5). Even with one 1% pellet there were among 14 animals of group IV only 4 with H.F. There was another striking phenomenon: *inhibition of cystic growth of Graafian follicles*. As already known they are in intrasplenic grafts, at two months, often very large (see column 7, group *) reaching an average diameter of about 1.5 mm. instead of about 1 mm. in the normal ovary (Gay 1944). With the administration of oestradiol the diameter of Graafian follicles drops considerably. Only in group IV, with but one 1% pellet, the diameter of these follicles reached and surpassed the normal size. The difference between the *group without oestradiol and the oestradiol groups I, II and III was highly significant. The average diameter drops to a level even beneath the normal of about 1 mm. (fig. 1). With two 1% pellets inhibition is already obtained as with pellets of pure oestradiol. If luteinization enhanced with similar pellets of pure oestradiol is counteracted with the simultaneous administration of progesterone the number of grafts with Graafian follicles attaining the normal diameter is seemingly greater; but the difference of the averages is scarcely significant.

Enhancement of *luteinization* by eostrogen is well known since long. It is spectacular with intrasplenic grafts when sufficient quantities of oestrogen are given (see group **, table 1, col. 6). In the present experiments with small, though variable, quantities of oestrogen (group I to IV), 25 out of 40 grafts had corpora lutea. The percentage of animals with corpora lutea was thus almost the same as in group * without oestrogen. Frequency of corpora lutea per animal and the average maximal diameter were seemingly somewhat increased but the difference was not singificant. Since a pronounced inhibition of cystic follicular growth was obtained with quantities of oestradiol yet unable to produce an enhancement of luteinization in groups I, II and III, these findings give new and more substantial evidence that the two phases of ovarian development—follicular growth and luteinization—are subject, if at all, to the control at very different quantitative levels of oestrogen.

DISCUSSION

How much oestradiol was absorbed from our pellets? The question is indeed crucial in our context.

Absorption from pellets of steroids mixed with cholesterol is not

selective (Shimkin and White 1941; Fuenzalida 1944); though the question has not been studied specifically for oestradiol it must be assumed that as to this the latter behaves similarly to other steroids. Absorption of oestrogen from mixed pellets is extremely slow. This has been proved by special experiments in which we made use of pellets which contained originally 1% of oestradiol (group V) but had been left in the body of guinea pigs for two years (from a series of experiments of Lipschutz and Riesco, unpublished). When again implanted into castrated guinea pigs for 45 days uterine weights of

TABLE 1. FORTY CASTRATED FEMALE GUINEA PIGS WITH AUTOPLASTIC INTRASPLENIC OVARIAN GRAFTS. TWO WEEKS AFTER TRANSPLANTATION A PELLET CONTAINING OESTRADIOL WAS IMPLANTED SUBCUTANEOUSLY

Group	Pellet, percentage of oestrad.	Supposedly absorbed per day μg.	Number of animals			Maximal diam. of Graaf. foll. group average mm.	Uterine weight g.
			Total	With blood foll.	With C.l.		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
I	10	9.0	9	0	5	0.49±0.08	3.1±0.25
II	5	4.5	12	0	8	0.62±0.06	3.4±0.41
III	1 two pellets	1.8	5	0	3	0.62±0.13	2.2±0.24
IV	1 one pellet	0.9	14	4	9	1.16±0.08	2.1±0.18
*	0	0	48	37	24	1.55±0.09	
**	100	28–65	12	0	12	0.48±0.11	
V	1	—	8	—	—	—	1.43±0.13

* From Lipschutz a.oth. (1948).
** From Lipschutz a.oth. (1948).

1.1 to 1.8 gr. with an average of 1.45±0.13 were obtained (table 1). This is less than with fresh 1% pellets (group IV); but in any case it proves that absorption from similar oestradiol pellets is not selective and that it is very slow.

There is another and quite convincing quantitative aspect. When a pellet containing a mixture of a specific steroid with cholesterol is implanted, absorption is forcibly from a reduced surface, according to the percentage of this steroid in the mixture. The total surface of our pellets was about 65 mm.; absorption of oestradiol from 1 sq. mm. is 1.4 μg. per day (Lipschutz and Vargas 1941; Fuenzalida 1950). About 90 μg. are absorbed from such a pellet of pure oestradiol; no more than 9.0, 4.5, 1.8 and 0.9 μg. of oestradiol could have thus been absorbed per day in group I to IV (col. 3, table 1), according to the pellet surface occupied by the specific steroid. The quantity absorbed was most probably less; a careful examination of absorption of steroids mixed with cholesterol shows that absorption per sq. mm.

of the specific steroid may drop as much as 5 and even 20 times (Fuenzalida 1950).

The absorption figures of column 3 have to be compared to what is known on the quantities of oestrogen secreted by the ovary in the normal guinea pig. In guinea pigs fed with CCl_4, i.e. with the intrahepatic inactivation transitorily, though probably not completely, suppressed an average of oestrogen equivalent up to about 13 to 14 μg. of oestrone daily appeared in the urine of a 15 days period (Furlong, Krichesky and Glass 1949). One may tentatively assume that more than that is entering into the general circulation. From this comparative point of view the quantities used in our experiments as given in column 3 of table 1 were certainly still much behind those in the normal animal. Thus a control of follicular development by oestrogen would still be, according to our results, within the range of physiological quantitative possibilities.

One might then suppose that the ovary, by secreting continuously a certain quantity of oestrogen, controls the anterior lobe as to its gonadotrophic function. But things are evidently much more complicated than that. Not only was the vaginal entrance open during the whole lapse of 45 days; not only were the nipples and mammary glands developed; there was also uterine bleeding in some animals in groups I, II and III. Uterine weights (column 8 of table 1) increased in all groups beyond normal, even with one 1% pellet not yet sufficient for the complete control of the hypophysis (group IV); there was even, in several animals, a slight degree of cystic glandular hyperplasia. All this proves that conditions were with our device not normal, not physiological. As already insisted upon, the figure of 13 to 14 μg. of oestrone per day referred to as appearing in the urine when the inactivating faculty of the liver is impaired is the *average* of a 15 days' period (Furlong *et al.* 1949). Much less than in our group III is probably secreted per day; the above mentioned high average of 13 to 14 μg. must be due to the sudden increase of oestrogen secretion in oestrus. This argumentation would be in favour of the assumption held by many authorities that ovarian control of the hypophysis is exercised not by the very small quantities of oestrogen continuously secreted but by the sudden increase of secretion in a given moment of the sexual cycle when large quantities of oestrogen enter into the general circulation.

It is remarkable that prevention of H.F. goes hand in hand with a diminished growth of Graafian follicles. One might feel inclined to conclude from this finding that follicular bleeding is due to uncontrolled and excessive follicular growth. But various other findings would be contrary to such a simple conclusion: (1) though H.F. in intrasplenic grafts are mostly of a considerable diameter *small* blood follicles also may occur; (2) purified extracts of hypophyseal FSH do not induce follicular bleeding (Li *et al.* 1949); (3) purified extracts

containing LH induce follicular bleeding (Claesson *et al.* 1948). One may then tentatively suggest that follicular bleeding is due to an abnormal timing of the secretion of the two gonadotrophins, this timing being controlled both by oestrogen and progesterone. Indeed, these assumptions presuppose that the control of follicular development is not due to a local action of both these steroids on the ovary (Iglesias, Lipschutz and Rojas 1950).

SUMMARY

Hemorrhagic follicles in intrasplenic ovarian grafts in castrated female guinea pigs are prevented already with quantities of α-oestradiol smaller than about 1.8 μg. per day absorbed from a subcutaneously implanted pellet.

Cystic growth of Graafian follicles also is inhibited by these quantities of oestrogen. With sufficient quantities of oestradiol the follicular diameter drops beneath normal.

Continuous absorption of even half the above quantities produces, in the course of several weeks, increase of uterine weight beyond normal and atypical epithelial proliferation of the endometrial glands.

One must then assume (1) that the quantities of oestrogen released continuously by the ovary of the guinea pig into the general circulation are smaller than the equivalent of 1.8 μg. of oestradiol, per day, and (2) that the control of follicular growth is due not to the action of similar minute quantities continuously released but by quantities probably immensely larger which are secreted at a given moment of the sex cycle.

Whereas control of follicular growth by oestrogen and enhancement of luteinization can be experimentally dissociated both these actions of the steroid being effected at different quantitative levels, the above statements make it not very likely that these actions are dissociated in the normal body.

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EXPERIMENTS WITH THE LOCAL ACTION OF OESTROGEN ON THE INTRASPLENIC OVARIAN GRAFT

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INTRODUCTION

PREVENTION of excessive follicular growth and of follicular bleeding in the intrasplenic ovarian graft, and enhancement of luteinization, have been produced in the castrated guinea pig to which oestrogen has been administered (Lipschutz a. oth. 1948; Barahona *et al.* 1950). These results have been interpreted, in accordance with the prevailing concept of the ovarian dynamics, as due to the action of oestrogen on the gonadotrophic function of the hypophysis. But it must be borne in mind that oestrogen has been found to cause ovarian changes also in hypophysectomized animals. Indeed, these actions of the hormone are not always coincident with those taking place in the intact animal; stimulation instead of inhibition of follicular growth in hypophysectomized rats has been reported by various authorities (Pencharz 1940; Williams 1940, 1944; Simpson *et al.* 1941). However, stimulation of follicular growth has been elicited by oestrogen also in intact immature rats (Gaarenstroom 1942; Gaarenstroom and De Jongh 1943). In hypophysectomized rabbits maintenance of corpora lutea was obtained by the administration of oestrogen (Westman and Jacobsohn 1937; Robson 1937), that is, again a result comparable to that generally observed in intact laboratory animals treated with oestrogen. But in hypophysectomized rats degeneration of corpora lutea was not prevented when oestrogen was given (Desclin 1949).

The above experimental statements make it necessary to examine the question whether a direct action of the administered oestrogen on the ovary might have interfered in our experiments with intrasplenic grafts and subcutaneously implanted pellets of oestrogen. Various workers tried to study the question of such a direct action on the ovary by implanting pellets of oestrogen into the rabbit's ovary in situ (Mayer 1948; Höhn and Robson 1949). But in similar experiments, as in the former ones with hypophysectomized animals, the oestrogen is allowed to enter into the general circulation and to act

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on other organs also, besides the ovary. The only way to avoid such an extraovarian action seemed to be the implantation of small oestrogen pellets into the ovary which subsequently is grafted into the spleen;[3] or the implantation of oestrogen pellets into the spleen in the proximity of the ovarian graft. As shall be explained below there is full evidence that under these experimental conditions oestrogen reaches the ovary whereas, as known since the work of Biskind, it does not enter into the general circulation.

PROCEDURE

Small pellets of α-estradiol weighing 0.6 to 2.4 mg. were introduced into the ovary and the latter was then grafted into the spleen of the same animal. The second ovary was removed. For implantation into the spleen pellets of about 25 mg. were used. They were introduced into the bottom of a tunnel made in the spleen; the ovary was introduced into the same tunnel and placed near the pellet. Necropsy took place 2 months after grafting. Material

TABLE 1. FORTY-TWO CASTRATED GUINEA PIGS WITH INTRASPLENIC OVARIAN AUTOGRAFTS. A SMALL PELLET WAS PLACED INTO THE OVARY BEFORE GRAFTING, OR A LARGE PELLET WAS IMPLANTED INTO THE SPLEEN BEHIND THE OVARY

Group	Pellet, percentage of oestradiol	Number of animals				Uterine weight gr.
		Total	With hem. foll.	With C.l.	With Graafian follicles	
	Intraovarian					
Ia	100*	2	0	0	1	1.7 (0.9–2.4)
IIa	90	5	1	0	5	0.9 (0.5–1.4)
IIIa	10	5	3	0	5	0.33 (0.25–0.4)
IVa	1	8	3	0	9	0.6 (0.3–0.8)
	Intrasplenic					
Ib	100	5	1	1	4	2.1 (1.1–4.1)
IIb	90	8	0	0	7	0.85 (0.8–1.4)
IIIb	10	3	3	0	3	0.7 (0.4–0.8)
IVb	1	6	3	2	5	0.3 (0.2–0.3)

* Pure oestradiol.

was fixed in Bouin's fluid. In 14 out of 26 experiments with the pellet in the ovary the latter was found when the spleen fixed in Bouin's fluid was examined macroscopically. With larger pellets implanted into the spleen the pellet was found in 21 out of 22 experiments.

EXPERIMENTAL RESULTS

Table 1 refers to 42 experiments only, those with adhesions having been omitted.

With 100 or 90% pellets only 2 out of 20 animals had hemorrhagic follicles (H.F.); on the contrary, with 10 and 1% pellets H.F. were found in 12 out of 22 animals. One might suppose, at first sight, that

[3] Höhn and Robson (1949) mention the urgency of this experimental procedure. The present work was undertaken in 1948, at the suggestion of R. Iglesias.

prevention of H.F. was due in the groups with 100 and 90% pellets to local action of oestrogen. But as shown in the last column of table 1, uterine weights increased considerably with 100 and 90% pellets. Nipples and mammary glands also developed. There was evidently an escape of oestradiol into the general circulation the inactivating faculty of the liver being a limited one (Lipschutz and Carrasco 1944). An escape of very small quantities into the general circulation would have been sufficient to cause inhibition of H.F. As previously found (Barahona *et al.* 1950) quantities so small as those which are absorbed from two subcutaneously implanted 1% pellets are already sufficient to prevent H.F. in the intrasplenic graft.

Results with intraovarian or intrasplenic pellets are compared in table 2 to those with subcutaneous pellets.

TABLE 2. COMPARATIVE ACTION OF SUBCUTANEOUS AND INTRASPLENIC PELLETS OF OESTRADIOL ON OVARIAN DEVELOPMENT

Site of pellet	Percentage of oestradiol in the pellet	Number of animals			Percentage	
		Total	With H.F.	With C.l.	With H.F.	With C.l.
Subcutan.*	10%, 5%, 1%	40	4	25	10	61
Intraovar.	10%, 1%	13	6†	0	54	9
Intraspl.	10%, 1%	9	6‡	2		

* Barahona, *et al.* (1950)
† *Pellet found in 2 of these 6 animals.*
‡ *Pellet found in all the 6 animals.*

Whereas percentage of H.F. was with subcutaneous pellets very small, percentage of H.F. was with intraovarian and intrasplenic pellets very considerable though not reaching the percentage one may obtain in intrasplenic grafts without the addition of oestrogen (Lipschutz *et al.* 1948).

DISCUSSION

Several objections can be made against the validity of the comparative figures given in table 2.

First: The 10 and 1% pellets implanted into the *ovary* were much smaller than those implanted subcutaneously and the quantities absorbed from intraovarian pellets might have been too small to prevent H.F. But this objection fails with pellets implanted into the *spleen* which were as large as those implanted subcutaneously; as known from former work (Lipschutz and Carrasco 1944) as much oestradiol is absorbed from intrasplenic pellets as from subcutaneous pellets. The quantity of oestradiol which reached the graft located near a pellet in the spleen must have been *many times* larger than that reaching the graft from a subcutaneous pellet.

Second: One might argue that in several cases the intraovarian pellet, or its cavity, could not be discovered at macroscopical or microscopical examination; but the pellet was present in all the

animals with intrasplenic grafts as given in table 2. In 8 out of the 12 animals with H.F. the ovarian or intrasplenic pellets were found, i.e., in these 8 animals H.F. were not prevented notwithstanding the pellets being present till the end of the experiment.

Third: An other objection might seem more relevant—how can we be sure that oestradiol absorbed from intraovarian or intrasplenic pellets had really reached the ovarian structures? That this was so is clearly evidenced by the fact that intraovarian and intrasplenic pellets unable to prevent H.F. counteracted luteinization. Whereas with

TABLE 3. COMPARATIVE ACTION OF SUBCUTANEOUS AND INTRASPLENIC PELLETS OF OESTRADIOL ON THE FOLLICULAR DIAMETER

Pellet, percentage of oestradiol	Subcutaneous* pellets	Intraovar. or intraspl. pellets	
	Foll. diam. mm.	No. of anim.	Foll. diam. mm.
100	0.48	7	0.83 ± 0.25
90	—	13	0.92 ± 0.16
10	0.49	8	0.95 ± 0.13
5	0.62	—	—
1	1.16	14	1.31 ± 0.07

* Barahona a.oth. 1950, table 1.

subcutaneously implanted mixed pellets luteinization goes on as before or is slightly stimulated, luteinization was mostly absent with intraovarian or intrasplenic pellets (table 1). In other words: oestradiol counteracts luteinization by direct action on the ovary, whereas it stimulates luteinization when reaching the general circulation. Prevention of luteinization by direct action on the ovary is very effective; even with intraovarian or intrasplenic 100 or 90% pellets when there was some escape of oestradiol into the general circulation, corpora lutea failed to appear; only 1 out of these 20 grafts had a corpus luteum (group I and II, table 1).

Growth of Graafian follicles is controlled by oestradiol absorbed from subcutaneously implanted pellets; with sufficient quantities even inhibition of follicular growth is obtained (Barahona a. oth. 1950). On the contrary, with intraovarian or intrasplenic pellets the follicular diameter was normal (table 3). Cystic growth was indeed counteracted. But the slight inhibition of follicular growth with intraovarian and intrasplenic 100% pellets was probably due to an escape of oestrogen into the general circulation. The fundamental difference between the behavior of the ovary according to the site of the pellet is exemplified by fig. 1.

It is a well known fact that ovarian "atrophy" is produced with the prolonged administration of large quantities of oestrogen both follicular development and luteinization being impaired (see for instance fig. E in Selye, 1947, p. 383). One might suppose that this

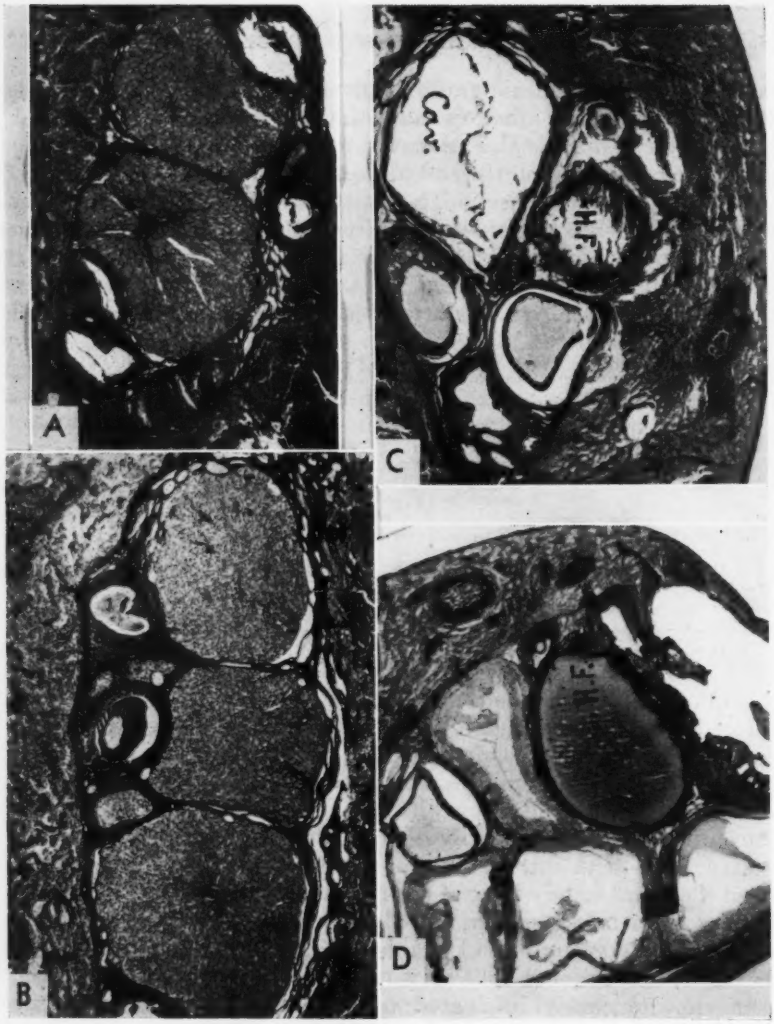

FIG. 1

Subcutaneous pellets:
Hemorrhagic follicles prevented.
Graafian foll. small.
Luteinization present; possibly enhanced.

Intraovarian & intrasplenic pellets:
Hemorrhagic follicles present.
Graafian foll. of normal size.
Luteinization prevented.

A. 5% pellet (CXXXIV.49). Absorption less than 4.5 μg. per day. Two corpora lutea. Various small Graafian foll.

B. 10% pellet (CXXXIV.84). Absorption less than 9 μg. per day. Three corpora lutea. Various small Graafian foll.

C. 10% pellet (CXXXV.92). Intraovarian. One hemorrhagic foll.; the diameter was in other sections greater than in the figured one. Graafian foll. of normal size. No corpora lutea. Cav.—cavity of the pellet.

D. 10% pellet (CXXXV.84). Intrasplenic. One hemorrhagic follicle. Graafian foll. of normal size. No corpora lutea. Cyst (of the rete?) on the right.

All figures at ×20.

condition is due to a *combined* direct and indirect action of oestrogen: (1) the quantity of oestrogen present in the general circulation is sufficiently large so as to cause a considerable decrease of the follicular diameter and simultaneously luteinization ought to be enhanced as in fig. 1A and 1B; (2) but the quantity of oestrogen which reaches the ovary is so great as to prevent this luteinization.

SUMMARY

Small pellets of oestradiol mixed with cholesterol have been implanted into the ovary of the castrated guinea pig and the ovary was then grafted into the spleen. In other experiments similar pellets were implanted into the spleen in the bottom of a tunnel, close to the ovarian graft.

With these pellets hemorrhagic follicles in the intrasplenic ovarian graft have not been prevented whereas with similar pellets when implanted subcutaneously prevention of hemorrhagic follicles was obtained.

With intraovarian or intrasplenic pellets the follicular diameter did not drop below the normal as with subcutaneous pellets.

Luteinization was almost completely prevented instead of being enhanced as with subcutaneous pellets.

In other words: when oestradiol was allowed to act directly on the ovary without being able to reach the general circulation the behaviour of the ovary was fundamentally different from that which was obtained with oestrogen reaching the general circulation.

From these comparative findings it is concluded that control of follicular growth and enhanced luteinization induced by oestrogen present in the general circulation are not due to a direct action of the hormone on the ovary.

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THE MATURATION OF THE PITUITARY-ADRENAL AXIS IN THE NEWBORN RAT[1]

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THE MATURATION OF THE PITUITARY-ADRENAL AXIS IN THE NEWBORN RAT

THE administration of adrenocorticotropic hormone (ACTH) results in an immediate fall in the ascorbic acid and cholesterol concentration of the adult rat and guinea pig adrenal gland (Sayers, Sayers, Fry, White and Long (1944); Sayers, Sayers, Liang and Long (1946)). The decline of ascorbic acid content becomes manifest within an hour after the injection of ACTH. There is also evidence that the fall in concentration of these substances is correlated with adrenal cortical secretion as shown by an increase in liver glycogen and lymphopenia. Selye (1937) had previously demonstrated that the administration of noxious substances resulted in adrenal hypertrophy in normal animals but not in hypophysectomized ones. He postulated that exposure to stress resulted in the release of ACTH by the animal's pituitary gland. Long and Fry (1945); Sayers and Sayers (1948); Ludewig and Chanutin (1947); Levin (1945) and others have shown that the adadministration of epinephrine, histamine or typhoid vaccine and exposure to cold, anoxia, vesicants, hemorrhage, etc., will also cause a decline in the adrenal cholesterol and ascorbic acid content of the rat. That this is mediated through the secretion of ACTH by the adenohypophysis is demonstrated by the fact that no alteration in the content of adrenal cholesterol and ascorbic acid occurs if the animal has been previously hypophysectomized. The injection of ACTH results in a decline of the cholesterol and ascorbic acid content of the adrenal regardless of the presence or absence of the animal's pituitary.

The purpose of the present study was to determine whether the pituitary-adrenal axis of the newborn was sufficiently mature to respond to stress.

METHODS AND MATERIALS

Pregnant rats of the Sherman strain were secured from a commercial breeder, brought into the laboratory, and kept in individual cages. They were

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allowed to deliver and the infant rats were nursed by the mother until the start of the experiment. Other animals of the same strain were bred in the laboratory. Only litter mates were used in each experiment, and males and females were approximately evenly divided between the experimental and control groups.

The experimental animals were injected with either 0.04 or 0.05 mg. epinephrine subcutaneously and killed between one and two hours later. Other animals were placed in the refrigerator at 5° for from 75 to 150 minutes. A third set of litters were given 0.05–2.5 mg. of Armour ACTH[2] subcutaneously and sacrificed 80 to 120 minutes later.

The animals were killed in two ways. Some were decapitated and the rest were injected intraperitoneally with Nembutal that had previously been warmed. This was done to insure the minimum amount of struggling and sudden death so that this would not constitute a stress in itself.

The adrenal glands were dissected free of connective tissue with fine iridectomy scissors and forceps under 5x magnification. It is more difficult to remove all the connective tissue from the very small adrenals than from the larger ones. Since the adrenal glands of infant rats are so small, usually 6 glands were pooled and weighed and analyzed. In the larger animals 4, and sometimes only 2, were used for the determination of ascorbic acid content. A slight modification of the method of Roe and Keuther (1943) using 8 instead of 15 ml. of trichloracetic acid was used for the estimation of ascorbic acid. In all, 232 infant rats from 36 litters were used in this study.

RESULTS

The adrenal ascorbic acid concentration of the infant rats is lower than that found in adults. There is some variation but the average was somewhat less than 300 mg. per cent as compared to over 400 mg. per cent for adult males.

EPINEPHRINE EXPERIMENTS

The injection of 0.05 mg. epinephrine subcutaneously resulted in about a 30 per cent mortality so that the dosage was reduced to 0.04 mg. which was toxic but sublethal. Within 2 minutes after the injection of the epinephrine the skin of the animals blanched and convulsions occurred for about one half hour. It is thus evident that the dosage of epinephrine was sufficient to act as a stress.

The results are shown in Figure 1. The administration of epinephrine does not result in a decline of adrenal ascorbic acid in 2, 4, and 6 day old rats. In animals 8 days of age, however, the same procedures resulted in a 31 per cent decrease. Animals 10 days of age responded to the epinephrine injection with an adrenal ascorbic acid decline of 34 per cent. Data obtained on adult male rats given 0.1 mg. epinephrine confirmed the results of Long and Fry (1945) and Sayers and Sayers (1948) in that there was almost a 40 per cent decrease in adrenal ascorbic acid.

[2] Kindly supplied by Dr. John R. Mote, Medical Director, Armour Laboratories.

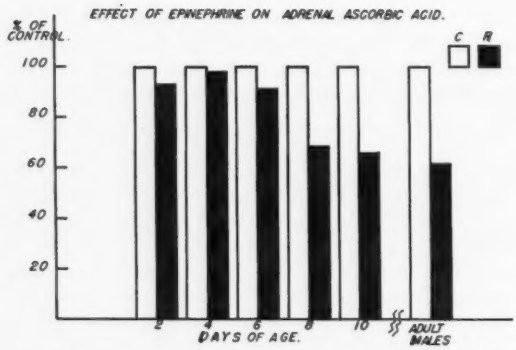

FIG. 1. The effect of epinephrine on adrenal ascorbic acid at various ages. The blank bars (c) represent the controls and the black bars (R_x) the injected animals.

EXPOSURE TO COLD

Rats of 4, 6, 7, 11, 14, 16, and 25 days of age were placed in the regrigerator (5° C) for from 75 to 150 minutes. This procedure caused no alteration in adrenal ascorbic acid until the animals had reached 16 days of age. In this age group, as in the 25 day old group, a reduction of 19 per cent occurred in the ascorbic acid concentration of the adrenal gland. The results are shown in Figure 2.

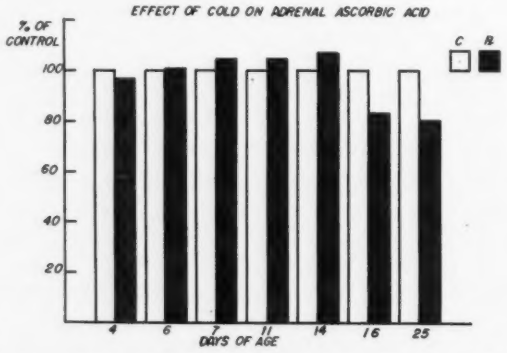

FIG. 2. The effect of cold on adrenal ascorbic acid at various ages. The blank bars (c) represent the controls and the black bars (R_x) the experimental animals.

ADMINISTRATION OF ACTH

Since the very young rats did not respond to the administration of epinephrine nor to exposure to cold, it was necessary to localize further the point of insensitivity, e.g. the pituitary or adrenal. Thus, infant rats 4 and 6 days of age were injected with 0.05–2.5 mg. of ACTH and sacrificed 80 to 120 minutes later. This resulted in a 24 to 37 per cent decline in the adrenal ascorbic acid in all animals. It

thus appears that the infant adrenal will respond to the injection of ACTH, under the conditions of these experiments. Figure 3.

OTHER NOXIOUS SUBSTANCES

The injection of several other noxious substances and tissue extracts failed to deplete the adrenal ascorbic acid in 3–4 day old rats. The administration of 0.1 unit pituitrin; 0.05 mg. histamine; normal human serum, saline extracts of brain, liver and spleen were without effect on the adrenal ascorbic acid content. The only tissue extract capable of causing a fall in the vitamin C content of the infant rat was pituitary.

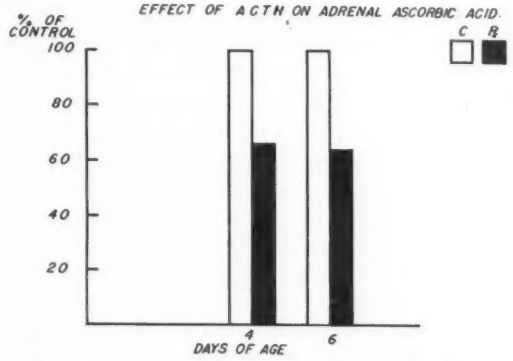

FIG. 3. The effect of subcutaneously administered ACTH on adrenal ascorbic acid. The symbols are the same as in the preceding figures.

DISCUSSION

Administration of epinephrine or subjection to stress produces depletion of adrenal ascorbic acid in an intact animal but produces no alteration in the vitamin C content of the adrenal of an animal that has been previously hypophysectomized. It is postulated that exposure to stress causes the adenohypophysis to secrete ACTH which in turn stimulates the adrenal cortex to increased secretion (Selye (1937), Long (1947), Sayers and Sayers (1948)).

However, the experiments cited in this report show that rats younger than 8 days of age do not respond to the administration of epinephrine as do older rats. Moon (1940) has previously demonstrated that the chronic administration of an impure adrenotrophic hormone will result in adrenal enlargement in 4 day old rats. The adrenal ascorbic acid declines as a result of the administration of one injection of ACTH. Therefore, the adrenal does have the capacity to respond to pituitary stimulation. It must be the pituitary that is not capable of responding to the epinephrine administration until the eighth day of life. Consequently, the response of the 4 day old rat, as

measured by a decline in adrenal ascorbic acid, should by a possible method of assay for ACTH. Experiments designed to test this are in progress.

Under the conditions of our experiments, exposure to cold did not cause a decline in adrenal ascorbic acid until the animals had reached the 16th day of life. This discrepancy in age may be explained by the fact that exposure to cold may not be as great a stress as the injection of 0.04 mg. epinephrine, since the rat does not become homiothermic until about the 14th day of life. Exposure to cold, therefore, may not not be a stress until the animal can regulate its own temperature. The younger rats when refrigerated become immobile and stiff and do not respond to painful stimuli. Sixteen day old animals remain conscious and mobile in the cold.

Several possible explanations can be offered to explain the phenomennn described.

1. If the stimulative action of epinephrine on the pituitary is mediated through a hypothalamic pituitary neural pathway, it is entirely possible that this pathway is not as yet myelinated in the rat below the age of 8 days. Therefore, epinephrine is without effect upon the pituitary.
2. There is evidence of increased corticoid secretion during pregnancy (Venning (1946)) and that the secretion of ACTH by the pituitary is regulated by the level of circulating adrenal cortical hormones (Sayers and Sayers (1948)). Since the adrenals of the newborn are hypertrophied at birth, the ACTH secreted by the maternal pituitary or other source must pass the placental barrier. It is possible that the level of circulating hormone in the newborn may be high and the action of epinephrine or cold on the pituitary is inhibited.
3. It is also possible that the newborn pituitary does not store sufficient ACTH so that even if stimulated it cannot respond by liberating any more ACTH.

Venning, Randall and Gyorgy (1949) have recently demonstrated that newborn human infants excrete small amounts of glucocorticoids and that severe trauma results in an increased output of these steroids. This has been interpreted as indicating that the pituitary is responding to stress by secreting ACTH. It would appear that the human is somewhat different from the rat. It should be emphasized, however, that the newborn rat is much less mature than the human. It is devoid of fur, it is poikilothermic and its eyes and ears are still closed. It is also possible that the rat anterior pituitary is less mature than the human pituitary.

SUMMARY

The administration of epinephrine to infant rats does not result in a fall of adrenal ascorbic acid until the rats are at least eight days of age.

Refrigeration, under the conditions of this experiment results in a fall in adrenal ascorbic acid only after the rats have attained the age of 16 days.

The administration of ACTH to rats four and six days of age causes a decrease in adrenal ascorbic acid. The possible implications of these results are discussed.

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IRON CONTENT AS A QUANTITATIVE MEASUREMENT OF THE EFFECT OF PREVIOUS PREGNANCIES ON THE MAMMARY GLANDS OF MICE[1]

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THE IRON retention that occurs in the epithelium of the mammary glands of mice has been pointed out (Schultz, '33; Rawlinson and Hankinson, '48) (figs. 1–3). Taking advantage of this it is possible to make whole-mount preparations of the glands using an iron stain and thus make a rough assessment of glandular development (Rawlinson, '50). It seemed likely that the determination of the iron content of such glands by histological and chemical methods would provide a measure of mammary gland development. It was with this object in view that the present experiment was planned.

It has been determined by Bittner (who kindly supplied the animals used to found the colony used in this study) that in his subline of C3H mice the cancer incidence in virgin females is 63% as compared with 95% in the breeding females, and that cancers occurred on the average about 4 months earlier in the latter group (Bittner and Huseby, '46). Another report (Huseby and Bittner, '46) shows that the mammary glands of animals used in breeding have a greater development of ducts, lateral buds, and hyperplastic nodules than the virgins. These findings point to a difference in the action of some factor in mammary development, presumably that of estrogenic stimulation. Consequently a comparative study of iron deposition in virgin and breeder C3H mice was chosen as a likely opportunity to determine to what extent the iron content would measure glandular development and thus, indirectly, the factors behind it.

METHODS

Three groups of C3H mice, aged 25 to 30 weeks were used. They consisted of males, virgins, and breeding females that had been isolated from the males for at least 4 weeks before death and had not been pregnant during this period. They were killed by decapitation and the skins removed and stretched out. The pair of inguinal gland pads were used for chemical analy-

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[2] G.B.P. is the holder of a Research Fellowship of the College of Physicians and Surgeons of Alberta.

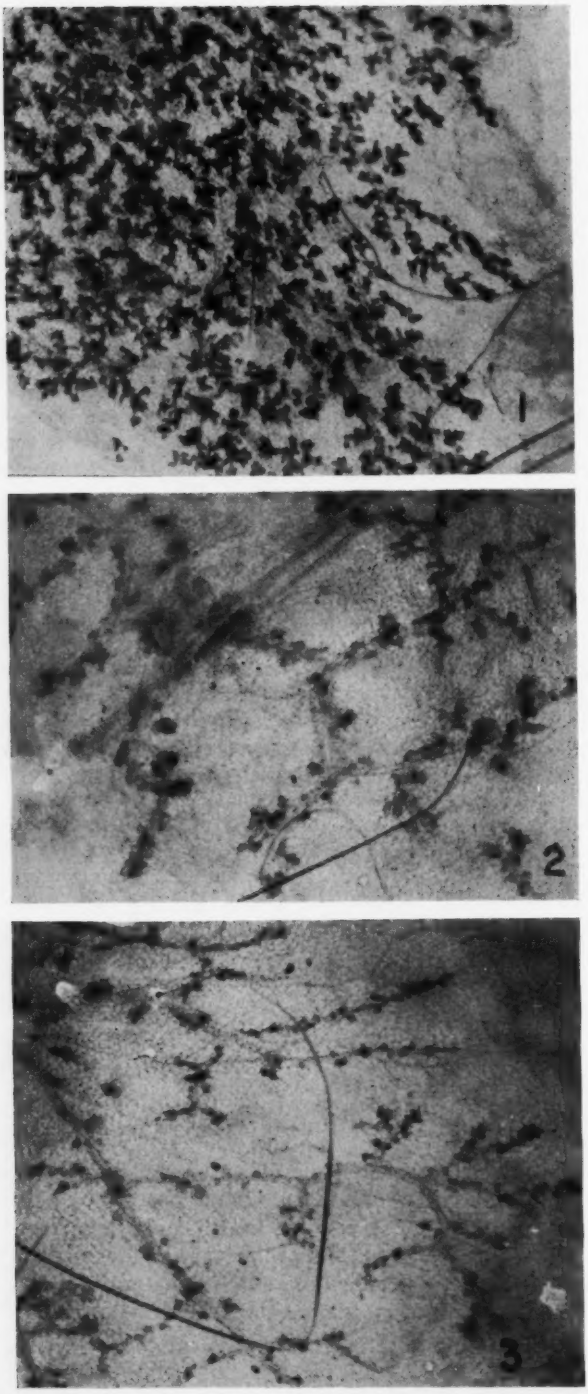
sis because it is possible to remove them practically as units and with very little extraneous tissue. Before the glands were freed, the lymph nodes belonging to them were shelled out. There is always a large one centrally placed in each gland and often a smaller one at the dorsal extremity. The removal of the nodes and the freeing of the glands from the hide were done with the aid of glass splinters that had been acid-washed to remove all traces of contaminant iron. The hide with the remaining mammary glands was fixed in buffered formalin for histological study using whole-gland mounts in which the iron was stained by the potassium ferrocyanide-hydrochloric acid method (Rawlinson, '50).

The removed inguinal tissue was weighed wet, ashed according to the method of Jackson ('38), and the iron determined by the use of a-a-dipyridyl (Hill, '31) on the basis of the technique of Kitzes *et al.* ('44). Because the amounts of iron to be determined were small it was necessary to exercise great care to avoid iron contamination. All the glassware used was thoroughly washed in a commercial detergent, rinsed, allowed to soak for two hours in concentrated HCl and rinsed 3 times in tap water and again 3 times in redistilled water; all reagents used were purified where possible to reduce reagent iron. The analytic procedure was as follows:

One ml. of conc. H_2SO_4 and 3 ml. of conc. HNO_3 were added to the tissue in a 30 ml pyrex micro-kjeldahl flask and heated until the gland tissue was dissolved: 1 ml. of perchloric acid was added and boiling continued and if charring occurred HNO_3 was added drop by drop until the solution cleared: heating was carried out until a water-clear solution was obtained, which was then cooled and diluted with 8 to 10 ml. of redistilled water. To measure the amount of iron obtained 1 drop of indicator (0.2% p-nitro-phenol in water) was added, followed by conc. NH_4OH until the solution became yellow: 2 ml. of acetic acid-acetate buffer (pH 4.5) were added, and 2 drops of thioglycolic acid (Eastman commercial grade) giving a final pH of 4.3: 1 ml. of a-a-dipyridyl (0.2% in 5% acetic acid solution) was added and the red-colored solution transferred to a volumetric flask and made up to 25 ml. with redistilled water. After standing for ½ hour it was transferred to a cuvette and read in a Coleman Jr. spectrophotometer at a wave length of 515 mμ against a blank solution of reagents that had been run simultaneously.

RESULTS

Histological: The glands from the axillary region which were stained for iron in whole mounts were graded on the basis of their histological iron content as 1, 2, or 3 plus, according as the stained iron in the gland cells appeared to be scant, moderate, or great in amount in the gland as a whole. The breeders all graded 3 plus with two exceptions, one of which was 2 plus and the other 1 plus. Of the virgins, 1 was listed as 3 plus, 7 as 2 plus, and 5 as 1 plus. The males showed no iron in their rudimentary mammary glands, but they all had stained iron in the lymph nodes associated with the glands. These results make it clear that even with this relatively crude measure of iron accumulation, the breeder mice tended to have appreciably more iron deposited in their mammary glands than did the virgins.



See facing page for explanation.

Chemical: The results of the chemical analysis are shown in Table 1, which gives the findings as total iron present in the glands and also as iron per gram of gland. The male mice were used to give some indication of the non-glandular iron in the mammary pad. The narrow range in the figures for the total iron in the males, and the small standard error, show that this iron was a fairly constant quantity. The iron in the virgin group exhibits a similar consistency. On the contrary, the figures for the breeding females have a wide range and

TABLE 1. THE IRON CONTENT OF THE TWO INGUINAL MAMMARY GLAND PADS OF C3H MICE AGED 25–30 WEEKS

Group	No. of mice	Total iron μg.			μg. iron/gr. of gland		
		Range	Mean	S.E.M.	Range	Mean	S.E.M.
Males	10	2.3– 5.4	4.0	0.3	15.3– 72.0	31.3	6.0
Virgin Females	13	4.5– 9.0	6.4	0.4	41.0– 92.0	60.9	4.6
Breeder Females	12	5.1–36.0	17.0	3.0	41.0–251.0	120.3	19.5

Statistical comparison of total iron content, or of iron per gram of gland, between any two of the three groups of animals gives a probability ratio of less than 1%. S.E.M. = Standard error of mean.

a much greater standard error. This lack of uniformity as compared with the virgins is to be expected since some of these animals had been less active in raising young than others in the group. No attempt was made to correlate the findings with individual breeding histories, but even on a purely random basis of selection that included animals that had been pregnant, regardless of the number of times, and whether they had raised their litters or not, the mean total iron is shown to be much greater in breeders than in virgins. Finally, a statistical comparison among the three groups demonstrated that not only is there a high degree of probability that true differences exist in total iron content between the males and each female group, but a similarly high degree of probability can be shown for the existence of a true difference between the virgins and breeders.

Table 1 also shows the results on the basis of iron per gram of gland. Here, although the ranges and the standard errors of the means are greater, there is again a high degree of consistency as regards

EXPLANATION OF FIGURES

FIG. 1. Whole mount of resting mammary gland of a multiparous C3H mouse, aged 30 weeks: stained for iron with potassium ferrocyanide-hydrochloric acid mixture and cleared without counterstain: the stained granules outline the peripheral portions of the gland tree: ×30: Iron graded histologically as 3 plus: analysis of inguinal glands, total iron 33 μg., 137.3 μg. per gram of gland.

FIGS. 2 and 3. Whole mount prepared and magnified as for fig. 1 from a virgin C3H mouse aged 30 weeks; figs. show two different areas of same gland with different stages of development: Iron graded histologically as 2 plus; analysis of inguinal glands, total iron 8.5 μg., 49.3 μg. per gram of gland.

males and virgins, and the same wide range in the breeders, yet once more statistical comparison gives a significantly high probability that true differences exist among the three groups.

Correlation of histological and chemical analysis. Table 2 shows the correlation between microscopic and chemical findings. In the three columns on the left the animals are arranged in descending order of total iron, on the right in order of iron per gram of gland. All his-

TABLE 2. RELATION BETWEEN CHEMICAL AND HISTOLOGICAL ESTIMATION OF IRON CONTENT OF THE TWO INGUINAL MAMMARY GLANDS IN VIRGIN AND BREEDER MICE

2a. Descending order of total iron: 2b. Descending order of iron per gram of gland

2a			2b		
Group	Total iron μg.	Hist. iron	Group	Iron/gm. gland μg.	Hist. iron
Br	37.0	3 plus	Br	250.6	3 plus
Br	33.0	3 plus	Br	228.1	3 plus
Br	24.5	3 plus	Br	162.6	3 plus
Br	20.0	3 plus	Br	137.3	3 plus
Br	18.0	3 plus	Br	124.7	3 plus
Br	17.0	3 plus	Br	120.8	3 plus
Br	14.0	3 plus	Br	120.4	3 plus
Br	14.0	3 plus	V	91.6	2 plus
V	9.0	2 plus	Br	87.1	3 plus
Br	9.0	3 plus	V	77.1	2 plus
V	8.5	2 plus	V	74.2	2 plus
V	7.5	2 plus	V	71.6	2 plus
V	7.5	2 plus	V	67.3	1 plus
V	7.5	3 plus	V	65.2	2 plus
Br	7.0	2 plus	Br	63.8	2 plus
Br	7.0	3 plus	V	63.1	3 plus
V	7.0	2 plus	Br	62.0	3 plus
V	6.5	2 plus	V	59.7	1 plus
V	6.5	1 plus	V	54.8	2 plus
V	6.0	1 plus	V	49.3	2 plus
Br	5.0	1 plus	V	48.1	1 plus
V	5.0	2 plus	Br	45.4	3 plus
V	5.0	1 plus	Br	40.9	1 plus
V	4.5	1 plus	V	40.6	1 plus
V	4.5	1 plus	V	28.5	1 plus

V = Virgins: Br = Breeders: Iron increases from 1 to 3 plus.

tological estimations were done independently without knowledge of the chemical analysis and confirmed by re-checking later at random. The males, which are not included in the table, never showed glandular histological iron and in them the average total iron (4.0 μg, Table 1) was below that of the lowest ranking virgin (4.5 μg, Table 2), while on the basis of iron per gram of gland the figures were 31.3 and 28.5 μg respectively. In each animal, the estimation of iron stained in whole gland mounts had to be done on different glands to those used for chemical analysis. In addition, the pattern and degree of alveolar development in any one mammary gland varies characteristically from

area to area (figs. 2 & 3). Therefore only an overall and roughly approximate grading of iron content can be made histologically. In the light of these considerations the degree of correlation is quite good.

DISCUSSION

An attempt to use the analysis of iron content as a measure of stainable iron in mammary gland cells presents peculiar difficulties. The main one is that of determining on what basis the results should be judged so as to make them as nearly as possible a measure of iron actually within epithelial cells. Since the lymph nodes of both male and female mice contain visible iron which is certainly non-glandular these were removed as a routine, especially as analysis of the nodes found in the two inguinal pads gave, on the average, about 2.0 μg. of iron per animal—a significant quantity in relation to the amounts involved in males and virgins, though of less consequence in the breeder group.

There still remains the iron that is present in solution in the tissue fluid, and also that bound to hemoglobin and other proteins and thus not reacting to the staining method used. This residual iron was reduced to a minimum by the free bleeding that followed decapitation, and the magnitude of that remaining was estimated from an analysis of mammary gland pads in the male after the lymph nodes were removed. The figures in Table 1 show that, under the conditions of the experiment, this was a relatively constant factor and of sufficiently small magnitude that, for the present purpose, it need not be separately considered. This assumption is supported by the fact that, when total iron is considered, if the iron in any of the females was graded under the microscope as two plus or three plus in amount, the corresponding iron content was outside the range found in the males (2.3–5.4) with only one exception (cf. Tables 1 and 2). The results on the basis of iron per gram of gland are not so clear-cut but a similar overall trend is unmistakable.

Another, and more difficult factor to allow for is the peculiar arrangement by which the resting mammary gland of the mouse is contained within an extensive fat matrix from which it cannot be mechanically separated. That this could under some circumstances be a significant source of error is shown by the observation that when animals were obviously sick before they were killed, or when postmortem examination showed liver abscesses grossly (presumably from mouse typhoid which is endemic in this colony) the weights of the two inguinal glands were invariably far out of the range of that found in healthy animals (Table 3). However, in these cases, as comparison of Tables 1 and 3 shows, the total iron found in the sick animals was well within the normal range for its group. On the contrary, the figures for iron on a gland weight basis were either at the extreme limit of the range for healthy animals of the particular group or very far beyond it.

This leads to the conclusion that the weight loss in the glands of a sick animal is largely due to loss of fat substance rather than of gland. This is borne out by the histological findings in that whole mounts of the glands of these sick animals showed what appeared to be normal amounts of iron for the particular group, but the gland tree containing the iron was compressed into a shrunken mass in which the fat was reduced to a minimal amount.

It is clear that this last source of error affects the figure for iron per gram of gland much more than that of the total iron. This raises the possibility that the total iron content of the glands in a group of animals of comparable age, considered in conjunction with the findings in the males, might be the best simple technique of estimating glandular iron, since it is evidently less subject to disease conditions in the animals. However, reference of the iron to a gland weight basis has the advantage of allowing to a limited extent for normal variations in gland size, but in this case precautions must be taken to exclude sick animals. Any mouse whose body weight, or weight of the gland pad, is markedly out of the normal range for its sex and age, or which exhibits liver abscesses or other definite autopsy findings should be excluded.

TABLE 3. EFFECT OF DISEASE ON GLAND WEIGHT AND IRON ANALYSIS

Animal	Gland weight gm.	Normal gland weight		Total iron μg.	Iron/ gm. gld. μg.
		Range	Mean		
Male	0.0325	0.0747–0.1794	0.1458	2.5	77.0
Male	0.0180			3.0	167.0
Virgin female	0.0523	0.0746–0.1723	0.1113	6.5	124.0
Virgin female	0.0535			5.5	103.0
Breeder female	0.0402	0.1115–0.1611	0.1395	16.7	415.5
Breeder female	0.0577			12.7	220.0

Each of the six animals listed in the table was excluded from the main experiment because of definite signs of disease: the figures for normal gland weight are included for comparison.

It might be that the relation of the iron content to a fat-free dry-weight basis would be of greater value in measuring glandular iron. We have considered the possibility and made some preliminary studies, but since the technique outlined gave highly significant differences which correlated well with the histological findings, we thought it unnecessary at this time to adopt a more complicated procedure with its greater possibilities of experimental error, especially when such small quantities of iron were involved.

From the foregoing considerations it appears that chemical iron determinations can be used as an indication of the amount of histological iron in the resting mammary glands of mice, and that when the

gland is more highly developed, as in breeders when compared to virgins in the particular strain and age group used in this experiment, the glandular development is reflected in the chemical findings. If this difference in the glands of these two groups of C3H mice is due to estrogenic stimulation, then the iron analysis is indirectly a measure of the action of this factor.

SUMMARY

An investigation is described in which the chemical analysis of the iron that accumulates in the epithelial cells of the resting mammary gland in female mice is used as a measure of glandular development. The application of this method to a study of breeder and virgin C3H mice of 25 to 30 weeks of age showed significant differences in iron content of the glands between the two groups and led to the conclusion that such a technique could be useful in studying problems of estrogenic activity.

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THE INFLUENCE OF ADRENAL CORTICAL HORMONES ON SENSITIVITY OF MICE TO IONIZING RADIATION[1]

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THE ABILITY to alter resistance of animals to ionizing radiation is now well established (Treadwell *et al.*, 1943, Graham and Graham, 1949, Patt *et al.*, 1949). However, the physiological processes that accomplish this end are totally obscured. This paper attempts to define the place of the adrenal cortex in radio-sensitivity or resistance.

The adrenal cortex is known to have a protective function from various kinds of trauma. It responds promptly to stress in many forms (Selye, 1947). Adrenalectomized animals are much more vulnerable to infection, toxins and mechanical trauma than their intact brothers (Perla and Marmorston, 1941). The administration of potent adrenal cortical extract to adrenal deficient individuals may restore them to a normal state of resistance but when given to normal animals, does not ordinarily endow a super immunity (Perla and Marmorston, 1941).

When we began this study, estradiol (Treadwell *et al.*, 1943) and foreign protein (Hektoen, 1918, Auer and Witherbee, 1921) given about 10 days prior to radiation were reported to have a protective action in animals. These observations have been confirmed (Graham and Graham, 1949). Because of their diverse nature and the time interval involved, the resistance conferred was regarded as a secondary effect i.e. foreign protein and estrogen acted as stimuli rather than by their own immediate effects. This view was strengthened by the fact that these same substances given immediately after radiation intensified the deleterious effect of x-ray (Treadwell *et al.*, 1943, Graham and Graham, 1949, Patt *et al.*, 1949). Stilbestrol was found to have an even more striking influence on the survival rate after diffuse radiation. When given to male mice 10 days prior to radiation it increased the mortality rate but on administration immediately after x-ray it protected them. Females were not appreciably influenced by this synthetic estrogen (Graham and Graham, 1949). There are a number of sites where these materials could act to modify radio sen-

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sitivity. The adrenal was regarded as one of the most promising.

The adrenal is profoundly influenced by ionizing rays, which is demonstrated by a number of findings after total body exposure. Patt *et al.* (1949) demonstrated an immediate reduction in cholesterol content of the adrenal within 3 to 4 hours after radiation and subsequent marked enlargement of the gland. The lipid is visibly depleted when observed histologically. Lawrence (1949) found that there is an increased 17-ketosteroid excretion in dogs. In adrenalectomized animals the thymicolymphatic atrophy is less severe and regeneration more rapid than in normals (Leblond and Segal, 1942, Dougherty and White, 1946, Straube *et al.*, 1949). The pituitary apparently mediates the roentgen effect on the adrenal since neither the early cholesterol depletion nor the subsequent hypertrophy occur in rats hypophysectomized 7 days before irradiation. However, animals in whom the pituitary has been removed are more vulnerable to the lethal effects of radiation (Patt *et al.*, 1948).

EXPERIMENTAL RESULTS

Our studies were carried out on young adult Swiss mice with an average weight of 20 gm. Total body radiation was delivered by 200 kv. x-ray under standard conditions and injection was by the intraperitoneal route. The survival rates are listed in Table 1.

One dose of adrenal cortical extract (ACE) (Lipo-adrenal cortical extract, Upjohn) 0.1 cc. given 10 days prior to 400r radiation was protective, while the same material given immediately afterwards was deleterious in the females and without effect in the males. The protection afforded by ACE 10 days before radiation at 400r is obviously relative since the same procedure at 600r did not modify the 100% mortality rate.

ACE 0.1 cc. given 4 hours before x-ray was without effect. ACE in the larger dose of 0.2 cc. given 4 hours before radiation and once daily thereafter for a week probably is deleterious since it was associated with an increased mortality rate.

Forty animals were radiated with 550r and followed by daily vaginal smears in the females and oral smears in the males. We have previously noted (Graham and Graham, 1949) that a characteristic change occurs in the desquamated cells of vulnerable mice prior to death. When these changes occurred in each animal, a single injection of ACE 0.1 cc. was given with little or no benefit. Of 20 males radiated, 10 showed the cellular response; of these, 5 were treated with ACE and one died, 5 were given a control injection of Wesson oil and 2 died. Of 20 females, all showed the cellular response; 10 received ACE and 9 died, 7 received Wesson oil and 5 died while 3 received no injection and all died.

Although the series was of necessity small, it is evident that no protection was conferred by Kendall's adrenal cortical extract since

TABLE 1. THE EFFECT OF VARIOUS AGENTS ON THE MORTALITY RATE FOLLOWING RADIATION

The time of injection is related to the time of radiation. Pre-rad. is before radiation, post-rad. is after radiation. The number of animals employed in each experiment is indicated in parentheses after the mortality rate. The controls listed here were radiated but not injected. ACE is Lipo adrenal extract (Upjohn). Kendall's adrenal cortical extract is an aqueous whole extract of adrenal cortex 150 gm./cc. containing 1:100,000 merthiolate. DOCA is desoxycorticosterone.

Compound	Dose	Time of administration	Amount x-ray	Mortality at 40 days	
				Male	Female
ACE	0.1 cc.	10 days pre-rad.	400r	16% (25)	4% (25)
ACE	0.1 cc.	10 days pre-rad.	400r	12% (25)	0% (25)
ACE	0.1 cc.	Immed. post-rad.	400r	28% (25)	60% (25)
Controls	—	—	400r	30% (227)	30% (220)
ACE	0.1 cc.	10 days pre-rad.	600r	100% (25)	100% (25)
Controls	—	—	600r	100% (30)	100% (30)
ACE	0.1 cc.	4 hrs. pre-rad.	550r	50% (10)	60% (10)
Controls	—	—	550r	50% (10)	60% (10)
ACE	0.2 cc.	4 hrs. pre-rad. and daily for 7 days	550r	100% (10)	100% (10)
Controls	—	—	550r	80% (10)	60% (10)
Kendall's adrenal cortical extract	1.0 cc.	4 hrs. pre-rad. and daily until death	550r		100% (5)
Controls	—	—	550r		100% (5)
ACE	0.1 cc.	Immed. after cellular reaction	550r	20% (5)	90% (10)
Controls	—	—	550r	40% (10)	100% (10)
DOCA	0.1 mg.	10 days pre-rad.	400r	16% (25)	20% (25)
DOCA	0.1 mg.	Immed. post-rad.	400r	52% (25)	52% (25)
Controls	—	—	400r	30% (227)	30% (220)
11-dehydroxy-corticosterone	0.2 mg.	4 hrs. pre-rad., immed. following x-ray and daily for one week	550r	90% (10)	70% (10)
Controls	—	—	550r	80% (10)	80% (10)
11-dehydroxy-corticosterone	2.0 mg.	Immed. after rad.	550r	80% (20)	92% (25)
Controls	—	—	550r	75% (8)	90% (47)
Stilbestrol	0.1 mg.	10 days pre-rad.	400r	82% (35)	42% (14)
Stilbestrol	0.1 mg.	Immed. post-rad.	400r	8% (25)	33% (24)
Estradiol benzoate	0.083 mg.	10 days pre-rad.	400r	5% (40)	3% (30)
Estradiol benzoate	0.083 mg.	Immed. post-rad.	400r	32% (34)	35% (17)
Testosterone propionate	1.25 mg.	10 days pre-rad.	400r	24% (25)	20% (24)
Testosterone propionate	1.25 mg.	Immed. post-rad.	400r	52% (25)	68% (25)
Horse serum	0.5 cc.	10 days pre-rad.	400r	0% (25)	0% (25)
Horse serum	0.5 cc.	Immed. post-rad.	400r	28% (25)	28% (25)
Horse serum	1.0 cc.	Immed. post-rad.	400r	56% (32)	50% (16)
Controls	—	—	400r	30% (227)	30% (220)

all 5 animals were dead by the 7th day. Desoxycorticosterone (DOCA) 0.1 mg. injected 10 days before radiation was without effect but when given immediately afterwards, the mortality rate was increased.

11-dehydrocorticosterone 0.2 mg. administered 4 hours before radiation, immediately after radiation and daily thereafter for one week

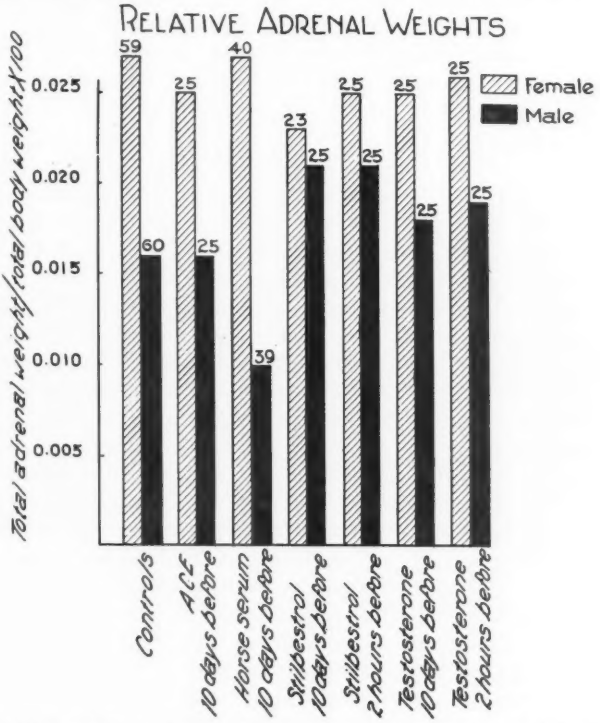

FIG. 1. The number at the top of each column indicates the number of animals in each group. ACE is Lipo-adrenal extract (Upjohn) 0.1 cc. The remaining amounts given were: horse serum 0.5 cc., stilbestrol 0.1 mg. in oil, testosterone propionate 1.25 mg. in oil. All injections were intraperitoneal.

increased the period of survival but did not alter the ultimate death rate. 11-dehydrocorticosterone 2.0 mg. was given immediately after radiation. All of the animals were temporarily anesthetized by the steroid but the survival rate was not modified.

In an attempt to correlate gross differences in the adrenal gland, a series of animals were injected with compounds which had produced changes in radiation sensitivity. Instead of radiating the animals, they were sacrificed and the adrenal gland removed on the day corresponding to the day of radiation in previous experiments. These animals received no radiation. The adrenals were weighed and are indicated in Figure 1. The size of the adrenal remains fairly constant

in the female. The male adrenal is significantly smaller 10 days after the administration of 0.5 cc. horse serum, but larger after giving stilbestrol 0.1 mg. either 2 hours or 10 days before. This effect of stilbestrol on the suprarenal is well known (Heskett and Huffman, 1943). The glands were then examined in paraffin section. An attempt was made to correlate the thickness of the glomerulosa, fasciculata and x-zones. Similarly they were studied in an effort to relate their histological appearance with the vulnerability to x-ray. However, neither measurements nor tissue detail showed deviation from the normal with sufficient regularity to justify any conclusion.

DISCUSSION

A direct relationship between the adrenal cortex and resistance to total body radiation has been postulated. Although this thesis cannot be decisively proved or rejected, a reasonable evaluation is possible from these and related published experiments.

Straube *et al.* (1949) and we have found that repeated injection of ACE aqueous or lipid, 17-dehydrocorticosterone or DOCA after radiation does not significantly modify the survival rate. Thus, it is to be doubted that increased adrenal secretion protects the animal from or intensifies the deleterious effects of radiation.

If liberal amounts of adrenal hormone are circulating during the stress, the depletion of adrenal cortex that ordinarily takes place is prevented (Sayers and Sayers, 1947). The possibility was entertained that if this principle were employed during x-ray the outcome might be altered. Swift *et al.* (1948) explored this field and found that they could prevent the early cholesterol loss in rats by administration of aqueous ACE but that the ultimate mortality was not modified. Similarly, we attempted to protect mice with a concentrated ACE in an oily medium and 11-dehydrocorticosterone but neither produced a significant effect on the survival rate. A buffering dose of ACE given just before radiation and then repeated daily thereafter not only failed to protect but may have diminished the resistance.

Adrenalectomized animals might be expected to succumb more readily if the suprarenal secretes a protective substance. On the other hand, if adrenal secretion were harmful, one might observe an increased survival rate after extirpation of the gland. Adrenalectomized rats (Patt *et al.*, 1947) and mice maintained on 1% NaCl drinking water show little difference in their mortality rate from intact animals.

The size and secretory activity of the gland at the time of radiation conceivably might condition the vulnerability of the adrenal and thus determine the ability of the animal to survive. A small adrenal is found in males after injection of foreign protein 10 days previously. This animal is highly resistant to radiation. However, the female similarly treated also enjoys the same resistance and the size of the suprarenal is unaltered by the horse serum. Again in the male, stil-

bestrol stimulates and causes the gland to enlarge. These animals are abnormally susceptible to the effects of x-ray. This fragmentary data suggests that a quiet, small gland at the time of radiation protects the animal. A more accurate evaluation would be possible with atrophy produced by repeated administration of adrenal hormone, and hypertrophy induced with adrenocorticotropic hormone (ACTH). Stilbestrol and ACTH both reduce the adrenal cortical cholesterol and Vitamin C, the former depletes the stainable lipids while the latter increases the sudanophilic material (Skelton *et al.*, 1949). However, the secretory activity and size of the gland may be of no more than academic interest in view of the remaining evidence that the adrenal has little influence on survival from radiation.

Adrenal cortical extract varies a good deal in its potency. Three different types have been tried; an aqueous extract (Wilson) by the Argonne group (Patt *et al.*, 1947, Straube *et al.*, 1949), an oily concentrated material (Upjohn) and another aqueous preparation (Kendall) all have failed to protect when given immediately before and/or repeatedly after radiation.

Ellinger (1947) has reported that desoxycorticosterone 0.5 mg. given repeatedly after radiation reduces the mortality rate. However, Straube *et al.* (1949) repeated the experiment and were unable to demonstrate a significant effect.

11-dehydrocorticosterone (Kendall's Compound A) is a glucocorticoid (as opposed to desoxycorticosterone, a mineralocorticoid), and so more closely concerned with resistance in general. In spite of this reputation it was without demonstrable effect as employed here. Although all of these preparations were of no value as supportive agents, it is entirely possible that some other product of the suprarenal may be.

Do adrenal cortical hormones have any effect on the resistance to radiation? Yes, a single injection of ACE 10 days prior to radiation protects both sexes. Because of the time interval, it is likely that the extract acted as a stimulus which if given sufficiently long before the trauma prepares the organism so that it can better withstand that stress. It is to be noted that desoxycorticosterone does not possess this property. We have no satisfactory explanation as to why ACE given immediately after radiation increases the radio sensitivity of females but not males.

SUMMARY AND CONCLUSION

Mice were given adrenal cortical extract, desoxycorticosterone and 11-dehydrocorticosterone at varying items both before and after total body radiation ranging from 400r to 600r.

We found that ACE given 10 days prior to radiation increases the survival rate in both sexes. When given immediately after radiation, ACE in the females increased the mortality rate. The remaining experiments did not alter the survival rate.

Although it is reasonable to postulate that the adrenal cortex plays a significant role in the resistance of an animal to radiation, our results do not confirm it.

We conclude that the resistance of an animal to total body radiation is not mediated by the adrenal cortical hormones here employed and probably not by the adrenal cortex itself. The demonstrable effects of adrenal compounds on radio resistance are in the nature of a non-specific stimulus since they may be duplicated by injection of other materials, e.g. foreign protein or estrogen.

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COMPARATIVE ACTIVITIES OF 17-HYDROXY-11-DESOXYCORTICOSTERONE AND 11-DESOXYCORTICOSTERONE[1]

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DESOXYCORTICOSTERONE acetate (DCA) corrects for some of the deficiencies caused by adrenalectomy. It also elicits functional and structural changes characteristic of hypertensive vascular disease (Selye *et al.*, 1943; Masson *et al.*, 1949). Its hormonal status in normal physiology is still in question. The possibility existed therefore that 17-hydroxy-11-desoxycorticosterone (compound "S")which has been isolated a so from the adrenals, might be the most significant natural salt-retaining hormone. Existing data on the pharmacological activities of this steroid are inadequate. Hence, we have compared the activity of its acetate with that of DCA under identical conditions in (1) adrenalectomized rats as well as in (2) rats under conditions used for the production of DCA hypertensive disease.

1. *Adrenalectomized rats.*

A. Experimental

28 rats weighing from 80 to 110 g. were adrenalectomized and divided equally into groups of 7. Group I consisted of untreated controls which received two daily injections of 0.1 cc. of cotton seed oil; group II, of rats treated with a daily dose of 0.15 mg. of DCA, and groups III and IV of rats which received respectively daily doses of 0.2 and 2 mg. of compound "S" acetate. These steroids were dissolved in oil; the daily doses of 0.2 cc. of oil solutions were administered in 2 subcutaneous injections of 0.1 cc. When 4 of the controls had died, animals of the other groups were sacrificed and blood taken for analysis of hematocrit index, blood urea nitrogen (Archibald, 1945) and plasma chloride (Van Slyke and Hiller, 1947).

B. Results

Data summarized in table 1 demonstrate that DCA and compound "S" acetate were both effective in life maintenance; the single deaths in each of the experimental groups occurred within 48 hours

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after adrenalectomy and are attributable to operative trauma. From the weight loss in group III (0.2 mg. compound "S" daily) it can be assumed that life could not have been maintained much longer. Weight gains occurred in groups II (DCA) and IV (2 mg. compound "S" daily). The animals of groups I and III all lost weight. Hemoconcentration, azotemia and hypochloremia due to adrenal insufficiency were prevented by treatment with 0.15 mg. of DCA and 2 mg. of compound "S", but 0.2 mg. of compound "S" held only the

TABLE 1. EFFECTS OF DCA AND COMPOUND "S" ON ADRENALECTOMIZED RATS

Group	Treatment and daily dose	Average body growth in g. on day			Mortality on 10th day	Hematocrit in per cent	Blood urea N mg. %	Blood chloride mg. %
		1st	5th	10th				
I	Oil	105	101	98	4	47 (45–49)	31 (26–39)	566 (555–578)
II	DCA 0.150 mg.	97	100	113	1	36 (30–40)	12 (8–14)	577 (566–584)
III	Cpd "S" 0.2 mg.	99	98	95	1	44 (40–50)	15 (14–18)	562 (526–572)
IV	Cpd "S" 2 mg.	90	97	112	1	35 (30–40)	12 (8–20)	589 (578–602)

azotemia in check. Briefly, a degree of functional maintenance can be obtained with a daily dose of 2 mg. of compound "S", but the same maintenance can be achieved with less than a tenth this amount of DCA.

2. *Unilaterally nephrectomized rats.*

A. Experimental

Since compound "S" seemed to possess qualitatively if not quantitatively the activity of DCA, it was of interest to investigate its effect on blood pressure. For that purpose, female rats weighing from 95 to 120 g. were divided into 4 groups of 5 animals each after unilateral nephrectomy. Animals of group I were kept as controls; those of group II received DCA, those of group III, compound "S" and those of group IV, DCA plus compound "S". These steroids were administered in the form of 30 mg. pellets implanted subcutaneously. Each rat of groups II and III received two pellets and each of group IV, four pellets. All the animals had Purina fox chow to eat and 1 per cent NaCl to drink. Blood pressure was taken regularly according to methods previously described (Kempf and Page, 1942; Olmsted *et al.*, 1948). Diuresis was also measured. On the 30th day, animals were sacrificed; tissues were fixed in Zenker's fluid, weighed and examined microscopically.

Pellet implantation was chosen because from previous experiments

with DCA, it was found an economic and effective way of eliciting hypertension. The validity of the comparison of pellet implants of different compounds may be brought in question. However, it has been shown that compound "S" is more water soluble than desoxycorticosterone because of its greater oxygenation (Reichstein and Shoppee, 1943). It should therefore be even more readily absorbed than DCA.

B. Results

As can be seen from fig. 1, blood pressure increased regularly in

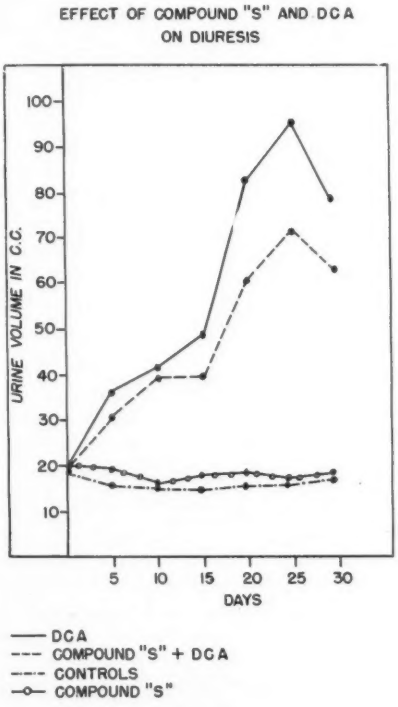

FIG. 1

groups II and IV, reaching values between 190 and 200 mm. Hg toward the end of the experiment; if we except a small plateau between the 10th and 15th day in the blood pressure curve of group IV, it is very similar to that obtained with DCA alone, indicating that compound "S" neither inhibits nor enhances hypertension. That compound "S" had no effect on blood pressure at the dose given is further demonstrated by the fact that the blood pressure curve of animals of group III follows very closely that of the untreated con-

trols (group I). Fig. 2 demonstrates that compound "S" does not affect diuresis as does DCA. Animals which received the combined treatment showed a slightly less diuresis than those receiving DCA alone; however, the group is too small for a valid conclusion. Rats

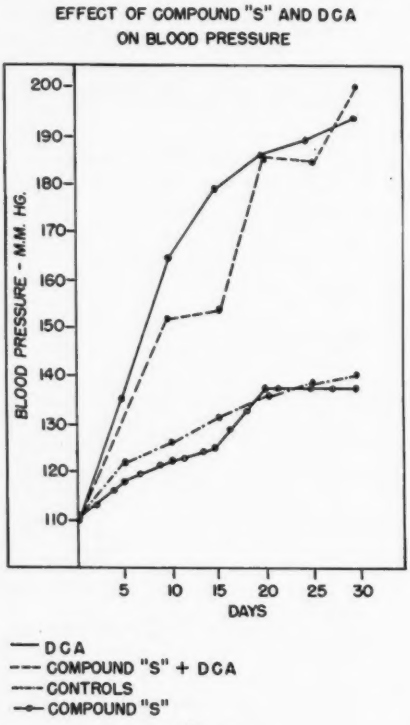

FIG. 2

which received compound "S" alone had the same diuresis as the controls.

In table 2 we give the average weight of various organs in the 4

TABLE 2. MORPHOLOGIC EFFECTS OF DCA AND COMPOUND "S" ACETATE

Group	Treatment	Body weight		Adrenal*	Thymus in mg.	Heart*	Kidney*	Arteritis, per cent incidence
		Initial	Final					
I	0	110	196	24 (22–29)	409 (361–447)	371 (340–413)	743 (682–811)	0
II	DCA	100	122	29 (24–34)	163 (120–246)	672 (443–1090)	1361 (1092–1809)	100
III	Cpd "S"	100	170	24 (18–28)	271 (237–293)	407 (364–479)	786 (655–900)	0
IV	DCA + Cpd "S"	107	161	25 (21–28)	244 (108–298)	463 (383–563)	1106 (942–1248)	57

* In mg. per 100 g. of body weight.

groups; adrenal, heart and kidney weights are expressed in per cent of body weight because of the large variations in this value. Kidney and heart weights were markedly increased in group II, but less so in group IV. Adrenals were stimulated in group II in spite of DCA treatment, as indicated by their slight increase in weight, and mainly by a decrease in thymus weight; the thymus was still smaller in groups II and IV which would suggest that compound "S" has some effect on sugar active steroid functions. At autopsy, macroscopic lesions of the kidney, heart and arteries were observed only in animals which received DCA alone or in combination. The incidence of lesions was less in group IV than in group II; however, whenever lesions were present, they were equally severe. Kidneys were pale, granular with multiple hemorrhagic points; hearts were hypertrophied with lesions appearing as white patches; splanchnic arteries were enlarged and nodular; and brains had cortical hemorrhages. Histological examination of these organs revealed changes characteristic of malignant hypertension in the rat (Masson, Hazard, Corcoran and Page, In Press). Therefore, it is not established that compound "S" had any inhibitory influence on DCA action, since final blood pressure and severity if not incidence of pathologic lesions were the same in groups II and IV.

In view of this uncertainty, we decided to repeat the experiment with a higher dosage of compound "S" acetate. The steroid was injected subcutaneously in a daily dose of 5 mg. as an aqueous suspension. There were 2 rats per group treated for 10 days. The DCA treated rats were hypertensive at the start of the experiment by reason of pellet implants. Six days after the beginning of the injections, the diuresis in rats treated with compound "S" alone increased from 22 to 36 cc., reached a maximum of 42 cc. on the 10th day, and came back to its original value of 20 cc. 6 days following discontinuation of treatment. Meanwhile, the diuresis of the control animals varied from 9 to 13 cc. Diuresis of the rats treated with DCA plus compound "S" did not differ from those receiving DCA alone. It can be assumed that the increased diuretic effect of compound "S" is more clearly demonstrable in normal animals with a basic diuresis of 22 cc. than in DCA treated rats with a diuresis of 150 cc. The blood pressure values at the end of treatment were 195 and 205 mm. Hg with DCA plus compound "S," 180 and 210 mm. Hg with DCA alone, 125 and 110 mm. Hg with compound "S" alone, and 118 and 126 mm. Hg in the untreated control rats.

DISCUSSION

Because of the presence of an hydroxyl group at C17, most of the work relative to the pharmacology of compound "S" concerned its possible action on the carbohydrate metabolism. However, this steroid did not enhance work performance of adrenalectomized rats, (Ingle, 1941) induce glycosuria in partially depancreatized rats, (Ingle, 1941)

or protect adrenalectomized mice against insulin shock (Reichstein and Shoppee, 1943). Its effect on electrolyte metabolism has been tested on one dog; it was found to possess a "low sodium and chloride-retaining" potency (Clinton and Thorn, 1942). This accords with the results obtained in the Everse-deFremery test which seems to be specific for this activity: 1 mg. of compound "S" was approximately as active as 0.08 mg. of DCA (Reichstein and Shoppee, 1943). Our results confirm these conclusions: using life maintenance, body growth and maintenance of normal blood chemistry as criteria of activity, we found that it has approximately one-thirteenth the activity of DCA. Experiments on unilaterally nephrectomized rats fed a high sodium diet showed also that the effect of compound "S" on sodium metabolism as indicated by diuresis, is very slight. If, as far as we know, the hypertensive properties of DCA are directly related to its effect on electrolyte metabolism, it was to be assumed that compound "S" would have little if any blood pressure raising effect. Our experiments confirm these views and furthermore show that it does not inhibit the hypertension elicited by DCA.

CONCLUSION

In adrenalectomized rats, the potency of compound "S" acetate was found to be approximately one thirteenth that of DCA. In unilaterally nephrectomized rats fed a high sodium diet, compound "S" acetate did not enhance or inhibit hypertension produced by DCA.

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DIFFERENT INTENSITIES OF SEXUAL ACTIVITY IN RELATION TO THE EFFECT OF TESTOSTERONE PROPIONATE IN THE MALE RABBIT[1]

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IT HAS been previously reported (Cheng and Casida 1949) that there is no consistent effect of testosterone propionate (dosages of 0.5, 1.0, 3.0, 10.0 and 20.0 mg. daily) on sperm concentration, total number of spermatozoa or volume of fluid portion of the semen in the rabbit. However, there was a significant increase in sexual libido and of seminal "glairy" substance in the treated animals over that in the controls. In the reported experiments the frequency of collections was held constant for any given dosage level.

Chang (1945) reported that by increasing the number of semen collections from rams there was an increase in the total number of sperm produced. At the same time there was a decrease in volume of the ejaculate, an increase in the time required to mate and some tendency for the concentration of the sperm in the semen to decrease.

The experiment reported here is an attempt to determine in the rabbit whether the effects of testosterone propionate (TP)[2] on semen production and sexual performance vary with different intensities of sexual activity (frequencies of collection).

EXPERIMENTAL PROCEDURE

Three groups of male rabbits (four animals each) were studied in the late winter of 1947–48. Each group was further divided into control and treated lots. The experimental interval of 32 days was divided into two equal periods. The information from the first of the two periods (which is called the pretreatment period or Pd I, a and c Table 1) gave an estimate of the rabbits' normal sexual activity, no hormone being administered to either lot within the group during this period. During the second period (which is called the treatment period or Pd II) the rabbits in the control lots (b, Table 1) received no hormone, while the rabbits of the treated lots (d, Table 1) received daily subcutanous injections of testosterone propionate. They received 10.0 mg. daily in 0.5 cc. pure corn oil.

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[2] Acknowledgment is made to the Ciba Pharmaceutical Products, Inc., for supplying the testosterone propionate for the conduct of this study.

Collections of semen were made by using an artificial vagina. The rabbits were trained to mount a dummy, which consisted of a dried rabbit skin wrapped over the arm of the operator, and to serve into the artificial vagina held in the hand under the dummy (Macirone and Walton, 1938). Six attempts to collect semen were made at half-hour intervals. The interval between days on which these attempts were made (collection days) varied in different groups of rabbits; four rabbits were collected from every day

TABLE 1. DESIGN OF EXPERIMENT AND METHOD OF ANALYSIS

Design of experiment

Lot	Pd I	Pd II
Control	a	b
Treated	c	d

a, b, c, or d = averages of all observations on the 2 animals of the lot for that period.
b-a = period effect
a-c = inherent lot differences
d-c = period effect plus treatment effect
(d-c) − (b-a) = treatment effect

Analysis of variance scheme

	Degrees of freedom		
	1-Day group	2-Day group	4-Day group
A. Period	1	1	1
B. Collection days within periods	30	14	6
C. Lots	1	1	1
D. Rabbits within lots	2	2	2
E. A×C (Treatment effects)	1	1	1
F. A×D	2	2	2
G. B×C	30	14	6
H. B×D (Error)	60	28	12
Total	127	63	31

(1-Day group), four every second day (2-Day group) and four every fourth day (4-Day group).

A stop watch was used to measure the length of time from the rabbits contact with the sexual stimulus (the "dummy" female) until the completion of the ejaculation, "reaction time." In the event that the rabbit failed to mount and ejaculate in 180 seconds, that attempt was considered a failure and the reaction time for that attempt was recorded as 180 seconds.

A hemocytometer was used to determine the concentration of sperm in the semen. One count was made on a sample from a composite of all semen from each rabbit for each collection day. A dilution of 1 part semen to 200 parts 4 per cent saline solution was used in making sperm counts. The diluted semen was put on a hemocytometer slide and the sperm counted under 450 magnifications of a microscope. An area of 1 mm. square was counted.

The volume of the semen for each attempt was measured and recorded. Rabbit semen contains a gelatinous material (called "glairy substance"), which is easily separated from the fluid portion of the seminal fluid. These two fractions were measured separately. The total number of sperm was determined by multiplying together the concentration of the composite sample and the volume of the fluid portion of the semen.

TABLE 2. EFFECTS OF TESTOSTERONE PROPIONATE IN THE DIFFERENT FREQUENCY-OF-COLLECTION GROUPS

Comparison		4-Day group collections made 1 day out of 4		2-Day group collections made 1 day out of 2		1-Day group collections made every day		F value for (between-group interaction with treatment effects)
Number of Ejaculates (Av./collection day)	C[1]	Pd I 6.0	Pd II 5.2	Pd I 5.6	Pd II 5.9	Pd I 4.2	Pd II 5.9	4.20*
	T[2]	5.6	6.0	5.7	6.0	4.7	6.0	
	T.E.[3]	1.2*		0		−0.4**		
Reaction time (Av. seconds/attempt)	C	42	69	57	38	97	46	2.04
	T	35	13	45	17	81	24	
	T.E.	49*		9		6**		
Volume semen (Av. cc./attempt)	C	.37	.31	.26	.22	.15	.11	0.78
	T	.31	.28	.29	.24	.13	.12	
Fluid portion only	T.E.	.03		−0.01		.03**		
Volume glairy sub. (Av. cc./attempt)	C	.20	.14	.31	.26	.13	.13	2.37
	T	.13	.43	.27	.38	.10	.28	
	T.E.	.36**		.16**		.18**		
Sperm concentration (Av. 1000/cmm./collection day)	C	390	262	238	262	141	136	0.17
	T	380	274	215	207	201	121	
	T.E.	22		−32		−75**		
Number of sperm (Av./attempt $\times 10^6$)	C	148	83	60	50	27	15	0.52
	T	105	78	73	51	30	15	
	T.E.	38		−12		−3		
Motility (Av. per cent/ejaculate)	C	74	64	65	52	63	45	0.08
	T	75	62	71	51	64	46	
	T.E.	−3		−7		0		

[1] Control lots.
[2] Treated lots.
[3] Treatment effect ((d-c) − (b-a), see table I).
* Signifies probability of chance occurrence <.05.
** Signifies probability of chance occurrence <.01.

An estimate was made of the per cent of the sperm showing progressive motility for each collection. The semen was first diluted with modified "Krebs" solution (Lardy and Phillips 1942) and the sperm were examined under high power magnification of a microscope.

The experimental design and the method of analysis are shown in Table 1. The letters a, b, c, and d represent the averages for all observations on 2 animals of a lot for the various periods. In the analysis for the effect of TP in each group, a–b represents the normal change in the untreated animals from Pd I to Pd II and thus is an estimate of the "period" effect in the treated animals. Sampling differences between the lots of animals is represented by a–c. The effect of treatment plus the normal change from Pd I to Pd II is given by d–c. The change from Pd I to Pd II in the treated animals over and above the normal change which would be expected if they had been untreated during that time is obtained from (d–c) − (b–a); this figure represents the net effect of treatment.

In the primary analysis of variance, A is the variation between Pd I and Pd II; B is the variation between collection days within the periods; C is the variation between the two lots and D is the variation between animals within lots. The interaction A×C is the differential change in the two lots from Pd I to Pd II and represents the effect of treatment. The interaction B×D is a

measure of the day-to-day variation in the differences between animals within lots and thus is the best estimate of experimental error.

In the analysis for the differences in the effect of TP under different frequencies of collection the data on the three groups were brought into a single analysis. The periods were divided into 4-Day intervals and the data from one collection day for each 4-Day interval was chosen at random. Thus all the data from the 4-Day group, one-half the data from the 2-Day group and one-fourth the data from the 1-Day group was used, except in the case of number of sperm, volume of semen (fluid portion) and volume of glairy substance where all the data were used for all groups. Significant differences in the effects of TP between the three frequencies of collection depended on the significance of the interaction between group, period and lot.

RESULTS

1. *Sexual Libido*

Libido, or sexual drive is indicated by the number of ejaculates obtained for the day and by the reaction time required for each attempt. In general the rabbits receiving hormone in each group had a shorter reaction time and a higher percentage of their attempts resulted in ejaculates.

In the 4-Day group, the individuals receiving TP gave on the average 0.4 more ejaculates per collection day during Pd II than during Pd I. The control animals gave 0.8 fewer ejaculates per collection day in the second period than in the first. This difference is significant and in favor of the treated animals.

The treated rabbits in the 2-Day group gave on the average 0.3 more ejaculates per collection day during Pd II than during Pd I. The control rabbits for this group, however, increased the same amount.

In the 1-Day group the control animals gave on the average 1.7 more ejaculates per collection day during Pd II than during Pd I, while the treated animals increased only 1.3 ejaculates per collection day. The difference is highly significant and in favor of the controls. It should be remembered that in the design of this experiment an individual was allowed a maximum of six attempts to ejaculate during a given collection day. All rabbits receiving the hormone during Pd II increased to the maximum number of six ejaculates per collection day. In no case did the control rabbits reach the same maximum during this period. This, in part, may then explain the lesser improvement from Pd I to Pd II in treated than in control animals in the 1-Day group, and it may also suggest that there might have been a greater difference between control and treated in the 2-Day and 4-Day group if the "ceiling" on the number of ejaculates per collection day had been higher. This was the only characteristic studied that showed a significant difference between the groups due to treatment; again however, any conclusion drawn could be erroneous due to the ceiling placed on the number of possible ejaculates.

The other indication of libido is the length of time required for the animals to react to the sexual stimuli by ejaculation. The reaction time for the rabbits receiving hormone in the 4-Day group decreased, on the average by 22 seconds per attempt, from Pd I to Pd II, while the control animals increased by 27 seconds for the same interval; the difference is significant. While the decrease in reaction time for the 2-Day treated group was on the average 28 seconds from Pd I to Pd II, the control animals decreased 19 seconds; the difference in this group was not significant. In the 1-Day group the decrease in reaction time for the treated rabbits was, on the average, 57 seconds from Pd I to Pd II; at the same time the control rabbits decreased only 51 seconds; the difference here is highly significant.

There was, on the average for all groups, a decrease of 14.3 seconds from Pd I to Pd II in the control rabbits and a decrease of 35.7 seconds in the rabbits receiving TP during the second period. There was, thus, a positive treatment effect on the average over all groups in the length of time required for the animals to react to the sexual stimuli, but the magnitude of this effect did not differ significantly among the groups.

2. *Seminal Material*

TP in all instances caused a highly significant increase in the volume of glairy substance, and again there was no evidence that the intensity of the effect differed with the frequency of collections.

TP caused a significantly smaller decrease in the volume of the fluid portion of the semen from Pd I to Pd II in the treated than occurred in the control lot for the 1-Day group. In this group the controls decreased, on the average 0.04 cc. per ejaculate, while those receiving TP decreased only 0.01 cc. In the 4-Day group and in the 2-Day group the controls decreased 0.06 cc. and 0.04 cc., while the treated rabbits decreased 0.03 cc. and 0.05 cc. respectively, and were not significantly different from the controls. Although the effect of testosterone was significant in the 1-Day group and not significant in the 2- and 4-Day groups, no significant difference could be shown between the groups in the intensity of effect.

3. *Sperm*

In the 1-Day group there was a greater decrease (highly significant) in sperm concentration from Pd I to Pd II, in the semen obtained from the treated rabbits, than in that from the controls. The controls decreased, on the average, 5,000 sperm for each cmm. of semen, while the treated animals decreased 80,000 per cmm. over the same interval. This was the only group that showed a significant treatment effect. In the 4-Day group of rabbits the control and treated lots decreased on the average 128,000 and 106,000 sperm per cmm. of semen, respectively; in the 2-Day group the control rabbits, on the

average, increased 24,000 while the treated rabbits decreased 8,000. The treatment effect on the concentration of sperm became numerically greater as the number of attempts for the experimental interval increased, with a range from very slight in the 4-Day group to highly significant in the 1-Day group. This difference in the treatment effect between the groups, however, was not great enough to be significant.

The difference in sperm concentration is probably not attributable to a direct effect of TP on the rate of spermatogenesis. There was a tendency for the volume of semen to increase with no significant change in the total number of sperm produced due to the hormone. It therefore appears that the lowering of the concentration of the sperm in the semen of the treated animals could simply be due to dilution of the sperm with the increased seminal fluid in those animals.

The change, from Pd I to Pd II, in the average number of sperm produced per attempt or in the progressive motility of those sperm for the animals receiving hormone was not significantly different from the change occurring in the control animals.

SUMMARY

Twelve rabbits were used to determine the effect of testosterone propionate on semen production and sexual libido for various frequencies of collection. The treated rabbits were injected with 10 mgs. of hormone daily. Attempts to collect semen were made six times over a period of three hours on each collection day. The intervals between collection days were one, two and four days.

In general the results of these experiments indicated that there is an increase in libido due to treatment. This is shown by an increase in the number of completed ejaculates by the males receiving the hormone and also by a decrease in the length of time required by the individual to react to the sexual stimuli. There was some increase in the volume of the fluid portion of the semen and a large increase in the glairy substance. A decrease in sperm concentration was observed but on the other hand there was no significant change in the total number of sperm. The lowering of the sperm concentration may be due to increased dilution with seminal fluid. The magnitude of the treatment effect did not in general seem to differ for the three frequencies of semen collection.

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THE EFFECT OF ANDROGEN ON SPERMATOGENESIS

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INTRODUCTION[1]

THE INHIBITING effects of the male sex hormone on testis weight and spermatogenesis in the rat have been reported by numerous workers using wide ranges of doses of androgens in various ages of animals. The trend of the results, however, shows an apparently anomalous situation in that small doses of androgen (0.1 to 1.0 mg. per day) caused greater injury than large doses (2.0 to 10.0 mg. per day) (Moore and Price, 1937, 1938; Selye and Friedman, 1941; Zahler, 1944; and numerous other references listed in the bibliographies of these papers). Administration of estrogenic substances to rats likewise produced a severe suppression of testicular function which could be prevented if androgens were injected simultaneously (Selye and Friedman, 1941; Gaarenstroom and deJongh, 1946).

The secretion of the gonadotrophins by the pituitary is apparently regulated by the circulating sex hormones, for the increased output of gonad-stimulating hormones following castration in the rat can be prevented by treatment with estrogens and androgens (Meyer, Leonard, Hisaw and Martin, 1930; Hamilton and Wolfe, 1938; Heller, Segaloff and Nelson, 1943). A comparable depression of gonadotrophic potency of the pituitary is associated with the testicular inhibition secured in normal rats injected with androgen (Moore and Price, 1937, 1938). The inhibiting action of the sex hormones on the testis is probably mediated through their suppression of the production of pituitary gonadotrophins.

In contrast to the inhibition of the testes of normal rats obtained with androgen injections, a stimulating effect on the testis has been observed in hypophysectomized rats with similar treatment. In the course of an experiment in which the effects of male sex hormone extracted from urine on the accessory sex glands of hypophysectomized adult male rats were being determined, Walsh, Cuyler and McCullagh (1934) made the amazing discovery that the injections of the hormone

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had maintained spermatogenesis in the testes and the weight of these organs was three times that of the hypophysectomized control rats. Nelson and Gallagher (1936) confirmed this announcement with extracts of androgen from urine. Following the purification, crystallization and synthesis of the androgens, other workers reported similar findings in the adult rat with synthetic substances (Nelson, 1937; Cutuly and Cutuly, 1940; Gaarenstroom and deJongh, 1946). Thus in the absence of the pituitary, spermatogenesis can be maintained with androgen.

Androgens exert a dual effect on the testis. By inhibiting the secretion of gonadotrophic hormones from the pituitary they serve to injure the seminiferous tubules in the normal animal. In the hypophysectomized male they maintain spermatogenesis, presumably by a direct stimulating effect on the testis, and in the estrogen-treated animal they counteract the deleterious effect of the female sex hormone on the testis by the same direct influence. It is probable that both of these effects occur when androgen is administered to the intact animal.

The experiments reported in this paper are designed to demonstrate the relationship between the size of the dose of testosterone propionate, the depressing influence on gonadotrophic potency of the pituitary and the direct stimulating effect on the testis. Results obtained with the administration of androgen to normal, hypophysectomized, and estrogen-injected rats and with the use of intratesticular implants of testosterone pellets are included in the evidence for the gonadotrophic action of androgen.

MATERIALS AND METHODS

Thirty-day-old and sixty-day-old rats of the Long-Evans strain averaging 66 and 166 grams in weight respectively were used throughout the experiment. All litters were raised in this laboratory and wherever possible littermates were used in setting up the following experimental groups.

Sixty-day-old Series

Untreated Controls

Testosterone Propionate Injections Daily for Thirty Days:

0.05, 0.1, 1.0 and 3.0 mg. in four groups of rats.

Thirty-day-old Series

Untreated Controls

Testosterone Propionate Injections Daily for Thirty Days:

0.01–0.04, 0.1, 1.0, 2.0 and 3.0 mg. in five groups of rats.

Estradiol Injections Daily for Thirty Days:

4.2 and 8.4 μg. in two groups of rats.

Combined Estradiol and Testosterone Propionate Injections Daily for Thirty Days:

4.2 μg. estradiol and 1.0 mg. testosterone propionate.

8.4 μg. estradiol and 1.0 mg. testosterone propionate.

Testosterone Pellets Implanted in Left Testis for Thirty Days:

100%, 15% and 5% pellets only in three groups of rats.

100%, 15% and 5% pellets and injections of 0.1 mg. testosterone propionate per day in three groups of rats.

Hypophysectomized Group:

Hypophysectomized Controls.

Hypophysectomized with injections of 1.0 mg. testosterone propionate per day for thirty days.

Daily subcutaneous injections of the hormones were administered in 0.1 cc. of peanut oil for a period of thirty days.[2]

Pellets of testosterone and testosterone propionate for intratesticular implants were prepared in this laboratory in 100%, 15% and 5% concentrations using cholesterol as a diluent. Thorough mixing of the ingredients was secured by cutting and piling the mound with a spatula on a smooth sheet of white paper. The pellets were shaped in a die which presses at one impression thirty-six cylindrical pellets 2 mm. in diameter. The weight of the pellets could be controlled by the amount of the mixture placed in the die. All pellets were sterilized with dry heat at 100° C. for one hour before they were administered. The propionate pellets failed to withstand sterilization and consequently were not used in the experiment.

Intratesticular pellet implants were made under ether anesthesia via an abdominal incision. The left testis was removed from the scrotum and the pellet inserted in the caudal end through a slit in the tunica albuginea. The abdominal adhesions which were prone to develop around this opening caused the testis to be retained in the body cavity and in subsequent operations the pellet was inserted in the cranial end of the testis with better results. The incision in the tunica was closed with a single silk suture. Those animals in which a cryptorchid testis resulted from the adhesions and those showing infection in the testis were discarded from the series at the time of autopsy. Some pellets were recovered at autopsy to determine the amount of hormone absorbed and others were left *in situ* to permit histological study of the local effects of the hormone on the seminiferous tubules. The recovered pellets were dried before they were weighed.

The hypophysectomies were performed by Dr. Warren O. Nelson by the parapharyngeal route under combined sodium amytal and ether anesthesia. Completeness of the operation was determined by checking the tissue in the suction flask at the time of operation and by careful inspection of the sella turcica at autopsy with histological section of any suspicious remnants. Incompletely hypophysectomized animals were discarded.

Autopsies were performed at the end of thirty days of treatment and at that time the testes, epididymides, an empty seminal vesicle, ventral prostate, adrenals, thymus, kidneys, and pituitary were removed and weighed to the nearest 0.1 mg. In those animals with extremely small accessory glands both seminal vesicles were weighed. A cross section of the testis in the region of the rete was routinely removed, fixed in Bouin's and stained in iron hematoxylin and eosin.

In order to determine the volume of sperm present in the epididymis, the head and tail were minced separately on a glass slide and two drops of 0.9% NaCl were added to form a sperm suspension. An estimate was then

[2] We are indebted to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Company for our supply of testosterone propionate and to Dr. J. Kenneth Thompson of Organon, Inc., for the alpha estradiol, testosterone and some commercial testosterone pellets.

made of the numbers and motility of the sperm by microscopic examination of the smears.

The majority of the pituitaries were dried in acetone and saved for assay, but the great decline in potency due to this treatment suggested that a better comparison of the gonadotrophic potency of the pituitaries of the various series of rats could be obtained if the use of acetone were avoided. Therefore the pituitaries from the last few groups of rats were assayed immediately or were macerated in 0.9% NaCl and frozen until they could be assayed. The acetone-dried pituitaries were powdered and dissolved in 0.9% NaCl made slightly alkaline with NaOH. The pH of the resulting solution was readjusted to neutrality with disodium phosphate.

All assays of the male pituitaries for gonadotrophic potency were carried out in immature female rats from twenty-five to thirty days of age. Two dried glands from the sixty-day-old series and three from the thirty-day-old series were administered to each female over a three-day period in a total of six injections and the rats were sacrificed twenty-four hours after the last injection. Ovaries and uteri were weighed and one ovary fixed in Bouin's. When fresh pituitaries were used, each female rat received one gland only.

Probable errors of the average weights of the testes, adrenals, pituitaries and kidneys and of body weight gain were computed for the various series of male rats. Variations in weights from those of the control animals were considered to be significant when the difference in weight exceeded the value of $4\sqrt{(PE_1)^2+(PE_2)^2}$.[3]

OBSERVATIONS

Sixty-day-old Series

Body Weight[4]

The control animals gained an average weight of 106 grams during the thirty days of the experiment and variations from this mean were seen in the averages for the four androgen-injected series. The rats receiving the 1.0 and 3.0 mg. of testosterone propionate per day showed weight gains which were less, though not significantly, than that of the controls, and the rats in the 0.05 and 0.1 mg. groups gained more weight than did the controls (table 1).

Organ Weights

Those organs of the body depending for their full growth and development on the blood levels of androgen, the prostate and the seminal vesicles, naturally showed an increase in weight in the treated rats, the augmentation being more or less commensurate with the dose. The weight of the thymus was decreased in the 1.0 and 3.0

[3] Reference text: *First Course in Statistical Method*, by G. I. Gavett. Criterion of statistical significance: "Unless a deviation is more than four times its probable error, there is no reason for thinking it may not result from pure chance" (p. 183).

[4] Complete data on body and organ weights of individual animals of all groups, together with statistical analyses and additional references, may be found in the doctoral thesis, "The Effect of Androgen on Spermatogenesis," by Dorothy Jensen, on file in the State University of Iowa library.

mg. groups but in the animals which received the lower doses of androgen the thymus was slightly larger than that of the controls (table 1).

The weight of the epididymis was less than that of the controls in the 0.1 mg. injected rats due to the decrease in the numbers of sperm present in the duct. Injections of 0.05 and 3.0 mg. of testosterone propionate resulted in slight increases in weight (table 1).

Inasmuch as various investigators have reported a renotrophic effect of the male sex hormone, the kidneys from all rats were weighed. A significant increase in weight with testosterone treatment occurred only in those groups receiving the high levels of androgen (1.0 mg., 11.74% increase over controls; 3.0 mg., 18.05% increase—table 1).

The injection of the various levels of androgen did not significantly alter the weight of either the pituitary or the adrenals (table 1).

TABLE 1. DATA CONCERNING AVERAGE BODY WEIGHTS AND ORGAN WEIGHTS OF SIXTY-DAY-OLD RATS INJECTED WITH DAILY DOSES OF TESTOSTERONE PROPIONATE FOR THIRTY DAYS AND OF CONTROLS OF THE SAME AGE

Series	No. of rats	Average body wt. grams			Empty sem. ves. mg.	Prostate mg.	Thymus mg.	Epididymis mg.	Adrenals mg.	Pituitary mg.	Kidneys	
		Initial	Final	Gain							Mg. (% body weight)	% difference from controls
90 day Controls	21	167	273	106	204.0	272.5	300.1	803.7	43.4	9.3	2533.8 (0.903 ± .020)	
.05 mg. t. prop.	5	179	302	123	208.8	347.8	320.8	855.7	48.9	10.1	2602.4 (0.862 ± .006)	−4.54%
0.1 mg. t. prop.	13	169	280	111	211.7	317.1	325.5	694.1	45.5	9.1	2672.0 (0.897 ± .012)	−0.66%
1.0 mg. t. prop.	17	161	249	88	335.9	512.3	172.2	753.4	39.9	8.2	2563.2 (1.009 ± .013)	11.74%*
3.0 mg. t. prop.	16	166	252	86	414.5	661.5	85.5	833.8	43.4	7.7	2680.5 (1.066 ± .022)	18.05%*

* Statistically significant.

The weight of the testes of the injected rats was less in all groups receiving androgen, but was least in the 0.1 mg. group (a statistically significant decrease of 24.71% compared to the controls). The testes of the 1.0 mg. injected rats likewise were significantly lighter (17.08%) than the controls. However, the smaller weights of the testes to be found in the 0.05 and 3.0 mg. groups (4.83% and 7.92% less than the controls) are not significant (table 2).

Histological Changes in the Testis

The microscopic picture of spermatogenesis in the testes of the androgen-treated animals showed no change from normal. Metamorphosing spermatids and mature sperm were found in nearly all of the seminiferous tubules. The Leydig cells were diminished in size and the quantity of the intertubular connective tissue appeared to be reduced following the injections.

Sperm were found in normal numbers and exhibited normal motility in the epididymis of all groups of rats with the exception of the 0.1 mg. group where they were present in somewhat reduced numbers and were less active.

Gonadotrophic Potency of the Pituitary

If the increase in weight of ovaries of immature female rats following the injection of a suspension of control pituitaries is used as a measure of maximum gonadotrophic potency (100%) and the gonadotrophin content of the pituitaries of the androgen-injected rats is calculated in terms of this standard, it is seen that all the dosage levels of testosterone propionate caused a significant decline in the ability of the pituitary to stimulate the immature ovary (table 2). The differences in potency of the pituitaries at the various dosage levels are, however, not significant in comparison with each other.

TABLE 2. DATA CONCERNING AVERAGE TESTIS WEIGHT AND GONADOTROPHIC POTENCY OF PITUITARIES OF SIXTY-DAY-OLD RATS INJECTED WITH DAILY DOSES OF TESTOSTERONE PROPIONATE FOR THIRTY DAYS AND OF UNTREATED CONTROLS OF THE SAME AGE

Series	Testis weight			Average weights of uterus and ovaries of immature female rats used in assay of dried pituitaries of sixty-day-old male rats (2 pituitaries per female)				
	Mg.	% body weight	% difference from controls	No. of females	Wt. fresh pit. mg.	Uterus mg.	Ovaries mg.	% of control potency
90 day Controls	2806.7	1.036 ± .020		6	18.0	89.9	89.1	100%
.05 mg. t. prop.	2971.9	.986 ± .008	−4.83%					
0.1 mg. t. prop.	2192.7	.780 ± .019	−24.71%*	3	17.5	96.7	41.8	31.15%
1.0 mg. t. prop.	2133.6	.859 ± .019	−17.08%*	7	15.9	56.7	29.1	12.66%
3.0 mg. t. prop.	2401.2	.954 ± .015	−7.92%	7	14.9	47.2	30.2	14.27%
Uninjected immature female controls				14		35.1	20.4	0%

* Statistically significant.

When the weight of the testes of these rats is compared with the gonadotrophic potency of their pituitaries (fig. 1) it is apparent that the low content of gonadotrophin in the pituitaries secured with the 1.0 mg. and 3.0 mg. doses of testosterone propionate (13% and 14% of control potency) accompanies heavier testes than does the higher pituitary potency (31%) which resulted from treatment of the rats with 0.1 mg. of testosterone propionate.

Thirty-day-old Series

Body Weight

The rats receiving injections of 1.0, 2.0 and 3.0 mg. of testosterone propionate per day gained less weight during the thirty days of the experiment than did untreated controls of the same age (table 3). This

inhibition of growth was statistically significant with the 2.0 and 3.0 mg. doses. Estradiol likewise significantly depressed growth of the rats resulting in weight gains which were 19.12% less in the treated rats with 4.2 μg. per day and 29.41% less with 8.4 μg. Administration of testosterone propionate with the estradiol permitted somewhat greater weight gains.

Miscellaneous Organ Weights

The weights of the seminal vesicles and prostate were increased in all androgen-treated groups except for the 0.01–.04 mg. group where a

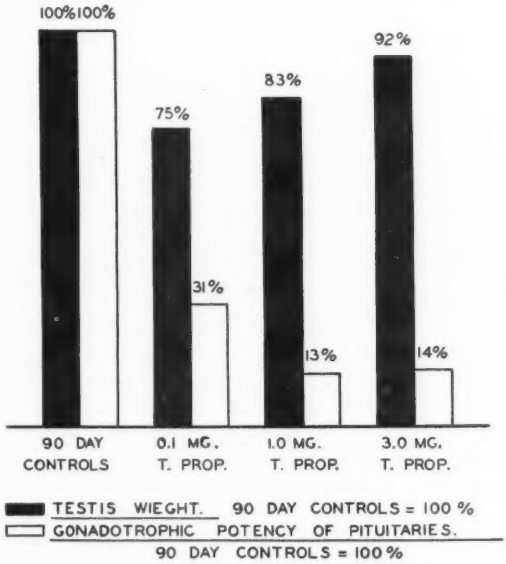

FIG. 1. Graph of testis weight and gonadotrophic potency of pituitaries (expressed as per cents of control data) of sixty-day-old rats used in the experiment.

slight decrease was noted (table 3). Increases were approximately in proportion to the dosage levels administered, though the 3.0 mg. group had glands weighing less than the 2.0 mg. series. Estradiol decreased the weights of the seminal vesicles and prostate markedly, but this effect was negated by the addition of 1.0 mg. of testosterone propionate per day and the weights of the glands compared favorably with those found in the rats treated with 1.0 mg. of testosterone propionate alone.

The weight of the thymus declined in all androgen-treated groups in proportion to the amount of testosterone propionate injected with the exception of the lowest dose (0.01–0.04 mg. per day). The weight of the thymus was not affected in the rats injected with 4.2 μg. of estradiol and only slightly decreased when the dose of estradiol was

TABLE 3. DATA CONCERNING AVERAGE BODY WEIGHTS AND ORGAN WEIGHTS OF THIRTY-DAY-OLD RATS INJECTED WITH DAILY DOSES OF TESTOSTERONE PROPIONATE, ESTRADIOL, OR BOTH FOR THIRTY DAYS AND OF CONTROLS OF THE SAME AGE

Series	No. of rats	Body weight				Empty sem. ves. mg.	Prostate mg.	Thymus mg.	Epididymis mg.
		Grams			% difference from controls				
		Initial	Final	Weight gain					
60 day Controls	38	68	204	136±2.5		96.0	140.8	388.0	367.8
.01–.04 mg. t. prop.	18	61	201	140±3.3	2.94%	76.5	119.4	467.2	305.8
0.1 mg. t. prop.	39	68	204	136±2.6		152.1	210.9	327.3	251.0
1.0 mg. t. prop.	33	64	190	126±1.9	−7.35%	299.5	409.5	174.1	391.6
2.0 mg. t. prop.	6	68	189	121±2.2	−11.03%*	347.2	476.3	159.5	494.5
3.0 mg. t. prop.	6	63	185	122±2.0	−10.29%*	289.5	394.8	139.5	478.3
4.2 μg. estradiol	10	68	178	110±3.1	−19.12%*	23.2	33.8	389.2	143.2
4.2 μg. e. and 1 mg. t. prop.	8	71	193	122±2.4	−10.29%*	357.3	457.3	140.3	523.2
8.4 μg. estradiol	6	76	172	96±3.4	−29.41%*	29.4	15.0	269.9	95.3
8.4 μg. e. and 1 mg. t. prop.	4	76	200	124±1.3	−8.82%*	324.3	474.6	115.2	534.5

* Statistically significant.

TABLE 4. DATA CONCERNING AVERAGE ADRENAL, PITUITARY AND KIDNEY WEIGHTS OF THIRTY-DAY-OLD RATS INJECTED WITH DAILY DOSES OF TESTOSTERONE PROPIONATE, ESTRADIOL, OR BOTH FOR THIRTY DAYS AND OF CONTROLS OF THE SAME AGE

Series	Adrenal weight		Pituitary weight		Kidney weight	
	Mg. (% body wt.)	% difference from controls	Mg. (% body wt.)	% difference from controls	Mg. (% body wt.)	% difference from controls
60 day Controls	35.6 (.0174±.0003)		6.6 (.00324±.00005)		2019.8 (0.994±.008)	
.01–.04 mg. t. prop.	37.1 (.0186±.0004)	6.90%	6.4 (.00322±.00012)	−0.62%	2064.1 (1.031±.011)	3.72%
0.1 mg. t. prop.	34.1 (.0168±.0002)	−3.45%	6.4 (.00315±.00006)	−2.78%	2144.5 (1.053±.010)	5.94%*
1.0 mg. t. prop.	33.9 (.0179±.0003)	2.87%	5.5 (.00293±.00006)	−9.57%	2020.8 (1.078±.013)	8.45%*
2.0 mg. t. prop.	37.1 (.0197±.0008)	13.22%	4.9 (.00259±.00008)	−20.06%*	1936.2 (1.027±.012)	3.32%
3.0 mg. t. prop.	33.5 (.0182±.0005)	4.60%	5.1 (.00278±.00004)	−14.20%*	1958.1 (1.061±.007)	6.74%*
4.2 μg. estradiol	50.0 (.0283±.0006)	62.64%*	7.7 (.00438±.00013)	35.18%*	1637.5 (0.921±.013)	−7.34%*
4.2 μg. e. and 1 mg. t. prop.	41.3 (.0214±.0004)	22.99%*	6.3 (.00328±.00004)	1.24%	2070.3 (1.075±.016)	8.15%*
8.4 μg. estradiol	56.4 (.0330±.0018)	89.65%*	9.3 (.00540±.00013)	66.67%*	1711.4 (0.996±.013)	−0.20%
8.4 μg. e. and 1 mg. t. prop.	51.3 (.0256±.0012)	47.13%*	5.6 (.00280±.00007)	−13.58%*	2364.4 (1.183±.035)	19.01%*

* Statistically significant.

doubled. The injection of 1.0 mg. of testosterone propionate with estradiol caused a marked fall in thymus weight (table 3).

The adrenals showed no significant differences in weight where testosterone propionate was administered alone (table 4). But 4.2 μg. of estradiol effected a significant increase in weight of 62.64% when given alone and a significant increase of 22.99% when 1.0 mg. of testosterone propionate was added. Where 8.4 μg. of estradiol were administered alone the adrenals of the rats were 89.65% heavier than the controls and 47.13% heavier with the addition of 1.0 mg. of testosterone propionate.

The weight of the pituitary was significantly less in the animals which received 2.0 and 3.0 mg. of testosterone propionate per day (20.06% and 14.20%) and significantly greater in the estradiol-treated rats (35.18% with 4.2 μg. and 66.67% with 8.4 μg.). The increase in weight in the estrogen-injected rats was prevented by the simultaneous administration of 1.0 mg. of testosterone propionate, and the combined treatment with testosterone propionate and 8.4 μg. of estradiol caused a significant decrease in weight of 13.58% (table 4).

Kidney weights were significantly increased in all testosterone propionate treated rats with the exception of the 0.01–0.04 mg. and the 2.0 mg. doses. The weight of the kidneys was significantly reduced by 4.2 μg. of estradiol (7.34%) but 8.4 μg. did not affect kidney weight. The simultaneous administration of 1.0 mg. of androgen and estradiol resulted in significant increases of 8.15% with 4.2 μg. and 19.01% with 8.4 μg. (table 4).

Testis Weight

The weight of the testes in all androgen-treated rats was significantly less than in the control animals (table 5). Lowest weights were found in the 0.1 mg. injected rats (64.75% less than controls). Estradiol exerted a similar inhibiting action on the testis weight (61.96% decrease with 4.2 μg. and 84.45% decrease with 8.4μg.). Large doses of androgen (1.0, 2.0 and 3.0 mg.) were less damaging, resulting in decreases from control weights of 25.78%, 14.96% and 11.24% respectively. The addition of 1.0 mg. of testosterone propionate to the estradiol injections protected the testis weight to some extent; decreases of 22.74% and 19.78% resulted from the combined treatment, reductions comparable to those obtained with 1.0 mg. of testosterone propionate alone.

Epididymis Weight

Epididymis weights were greatest in those rats which received heavy doses of testosterone propionate (table 3) and the sperm counts and motility were normal in these groups. Low doses of androgen (0.01–0.04 and 0.1 mg. per day) and injections of estradiol caused a decrease in epididymis weight, associated in each case with either a

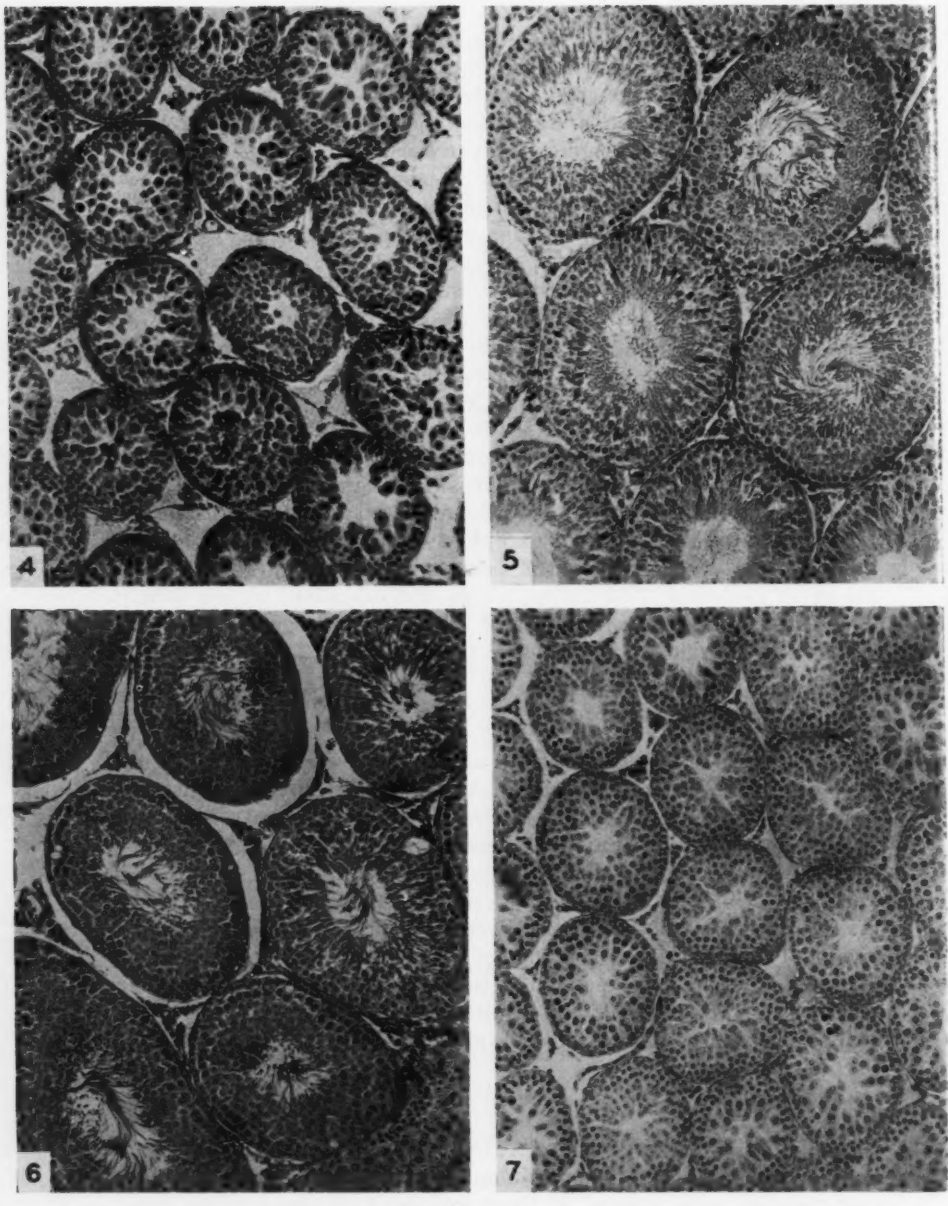

PLATE 1

complete absence of sperm or a reduction in numbers and motility. Combined doses of estradiol and testosterone propionate maintained the epididymis weight at above normal levels and the sperm population was similar to that found when 1.0 mg. of androgen was injected alone.

Histology of the Testis

Study of sections of the testis taken from these various groups revealed normal spermatogenesis in all of the androgen-treated groups with the exception of those rats injected with 0.1 mg. of testosterone propionate per day (fig. 4–8). The tubules of the testes of the rats receiving 0.01–0.04, 1.0 (fig. 8), 2.0 and 3.0 mg. of testosterone propionate per day were slightly smaller than those found in the testes of normal controls, but the predominant stages of spermatogenesis were the same—metamorphosing spermatids and mature sperm being found in nearly all of the tubules. The testes of the 0.1 mg. group were similar to those of rats sacrificed at the beginning of the experiment (thirty days of age). The tubules were small and the most advanced stages of spermatogenesis were secondary spermatocytes and spermatids (fig. 4 and 7).

Rats treated with estradiol alone had testes which were inhibited to the same extent as those receiving 0.1 mg. testosterone propionate (fig. 9). Here, too, the process of sperm formation was arrested at the spermatocyte or spermatid stage. The heavier dose of estradiol produced an inhibition comparable to that found in hypophysectomized rats, with spermatogonia and primary spermatocytes the only stages to be found in the tubules. The addition of 1.0 mg. of testosterone propionate to this treatment resulted in a normal histological picture, with metamorphosing spermatids and mature sperm in the tubules (fig. 10).

The interstitial cells were not appreciably altered by the lowest doses of testosterone propionate, but 0.1, 1.0, 2.0 and 3.0 mg. of testosterone propionate per day reduced the size of these cells and

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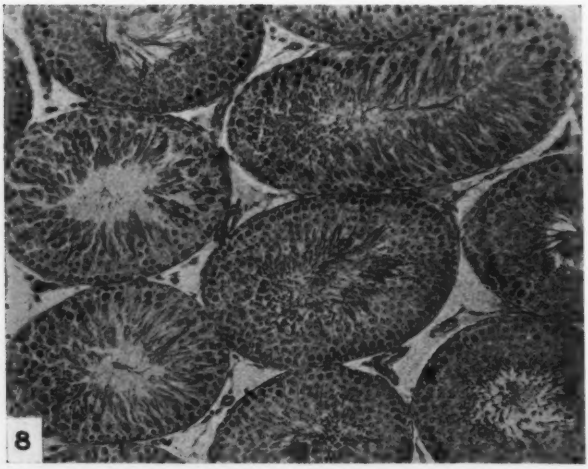
Photomicrographs of rat testes fixed in Bouin's, sectioned at 7μ, and stained in Regaud's hematoxylin and eosin. All sections photographed with 16 mm. objective and 7.5× ocular.

FIG. 4. Testis of thirty-day-old control rat showing the predominance of spermatocytes and spermatids found in the tubules at this age. Weight of testes, 553.2 mg.

FIG. 5. Testis of sixty-day-old control rat. Note increased tubule size and presence of sperm, compared with figure 4. Weight of testes, 2429.6 mg.

FIG. 6. Testis of thirty-day-old rat injected with 0.01 mg. of testosterone propionate per day for thirty days. Note slight reduction in tubule size (compare with figure 5), but no inhibition of spermatogenesis. Weight of testes, 2220.6 mg.

FIG. 7. Testis of thirty-day-old rat injected with 0.1 mg. of testosterone propionate per day for thirty days. Comparison with testis of sixty-day-old control (figure 5) shows inhibition of spermatogenesis and suppression of tubule growth secured with this dose. Note striking similarity to testis of thirty-day-old control (figure 4). Weight of testes, 461.0 mg.



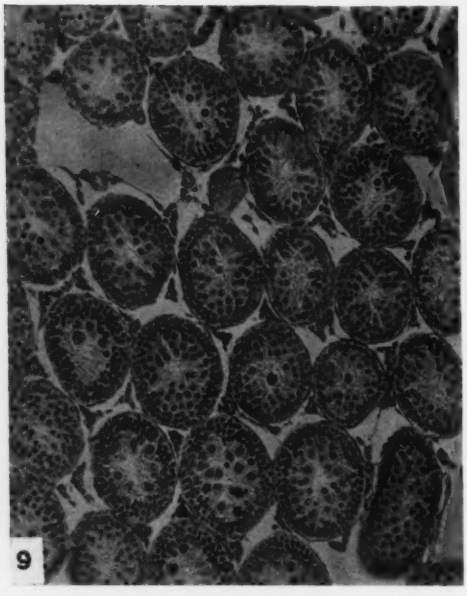


PLATE 2

FIG. 8. Testis of thirty-day-old rat injected with 1.0 mg. of testosterone propionate per day for thirty days. Note slight reduction of tubule size compared with that of normal sixty-day-old rat (figure 5) but lack of effect on sperm formation. Weight of testes, 1678.8 mg.

FIG. 9. Testis of thirty-day-old rat treated with 8.4 μg. of estradiol per day for thirty days. Note marked inhibition of spermatogenesis compared with that obtained with 0.1 mg. of testosterone propionate (figure 7). Weight of testes, 260.8 mg.

FIG. 10. Testis of thirty-day-old rat receiving 8.4 μg. of estradiol and 1.0 mg. of testosterone propionate per day for thirty days. Note maintenance of near normal tubule size and spermatogenesis with this combined treatment. Weight of testes, 2305.6 mg.

TABLE 5. DATA CONCERNING AVERAGE TESTIS WEIGHT AND GONADOTROPHIC POTENCY OF PITUITARIES OF THIRTY-DAY-OLD RATS INJECTED WITH DAILY DOSES OF TESTOSTERONE PROPIONATE, ESTRADIOL, OR BOTH FOR THIRTY DAYS AND OF CONTROLS OF THE SAME AGE

Series	Testis weight			Average weights of uterus and ovaries of immature female rats used in assay of dried pituitaries of thirty-day-old male rats (3 pituitaries per female)					Average weight of uterus and ovaries of immature female rats used in assay of fresh pituitaries of thirty-day-old male rats (1 pituitary per female)				
	Mg.	% body weight	% difference from controls	No. of females	Wt. fresh pit. mg.	Uterus mg.	Ovaries mg.	% of control potency	No. of females	Wt. fresh pit. mg.	Uterus mg.	Ovaries mg.	% of control potency
60 day Controls	2409.5	1.183 ± .012		6	19.9	80.9	83.6	100%	7	7.6	104.5	93.7	100%
.01–.04 mg. t. prop.	1917.2	.944 ± .044	−20.20%*	2	20.7	84.1	74.8	86.08%	3	5.9	126.2	62.3	57.16%
0.1 mg. t. prop.	876.7	.417 ± .021	−64.75%*	7	17.8	130.0	51.0	48.42%	2	7.7	109.1	47.0	36.29%
1.0 mg. t. prop.	1659.0	.878 ± .020	−25.78%*	3	17.3	31.7	22.1	2.69%	6	5.0	36.3	25.5	6.96%
2.0 mg. t. prop.	1897.2	1.006 ± .016	−14.96%*	2	14.7	56.5	23.8	5.38%					
3.0 mg. t. prop.	1857.8	1.050 ± .021	−11.24%*						6	5.1	36.0	19.1	0%
4.2 μg. estradiol	824.4	.450 ± .053	−61.96%*										
4.2 μg. e. and 1 mg. t. prop.	1763.7	.914 ± .030	−22.74%*										
8.4 μg. estradiol	319.9	.184 ± .010	−84.45%*						6	9.3	40.0	18.5	0%
8.4 μg. e. and 1 mg. t. prop.	1903.2	.949 ± .035	−19.78%*						5	5.4	37.2	23.4	4.09%
30-day-old rats	577.2	.764 ± .025				Uninjected immature female controls			14		35.1	20.4	0%

* Statistically significant.

caused their nuclei to become pycnotic (fig. 7 and 8). In some cases the interstitial cells were virtually indistinguishable from other intertubular connective tissue elements. The effect of estradiol and of the combined administration of estradiol and testosterone propionate was identical with that of the heavier concentrations of androgen, and the volume of the interstitial tissue was diminished in all cases (fig. 9 and 10).

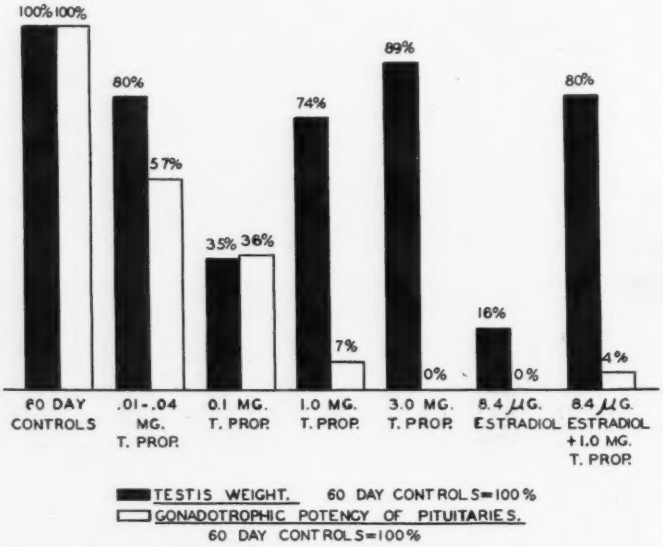


FIG. 2. Graph of testis weight and gonadotrophic potency of pituitaries (expressed as per cents of control data) of thirty-day-old rats used in the experiment.

Gonadotrophic Potency of the Pituitaries

Two different methods were used in assaying the pituitaries of the thirty-day-old series. The majority of the glands were acetone-dried and three pituitaries were injected into each female rat. The pituitaries from the last few groups were assayed as fresh material, one to a female rat. The trend of the results was similar with both techniques.

The lowest dose of androgen reduced the potency of the pituitaries to 86.08% (57.16%, fresh) of the control potency and each succeeding increase in dose further decreased the potency until no stimulation of the ovaries of the immature females was secured with pituitaries from the male rats which received 3.0 mg. of testosterone propionate per day (table 5).

A comparison of the testis weights with the gonadotrophic potency of the pituitary shows that those rats receiving the highest levels of androgen had the largest testes in spite of the fact that the decline in pituitary gonadotrophic potency was the greatest in those rats (table

5, fig. 2). The decrease in potency of the pituitaries of the rats receiving 0.01–0.04 mg. of testosterone propionate per day was not marked, but the testes of the rats were 20.20% lighter than the controls. Injection of 0.1 mg. of testosterone propionate had the most deleterious effect on the weight of the testes (a decrease of 64.75%) which was associated with a pituitary gonadotrophic potency slightly less than half that of the controls.

Estradiol was likewise effective in diminishing the gonadotrophic content of the pituitary (table 5, fig. 2). The testes of the rats treated with estradiol alone (8.4 μg.) were inhibited to the greatest extent (84.45% smaller than controls) of any in the thirty-day-old series, and the pituitaries of these rats were depleted of their gonadotrophic potency. The addition of 1.0 mg. of testosterone propionate to this dose of estradiol did not significantly increase the potency of the pituitaries but the testes of the rats were much less inhibited, being only 19.78% smaller than those of untreated controls.

Hypophysectomized Series

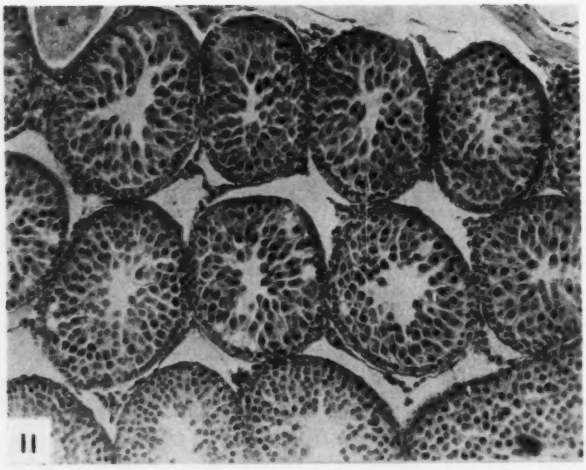
The data secured in this portion of the experiment were insufficient to be published at this time but the trend of the results indicated that androgen can stimulate spermatogenesis in thirty-day-old rats in the complete absence of the pituitary. The weight of the testes of the rats hypophysectomized for thirty days was 68% lower than that of a normal thirty-day-old rat and the injection of 1.0 mg. of testosterone propionate per day reduced this decline to 34% below the normal testis weight at thirty days. The weight of the epididymis was greatly increased in the androgen-treated rats, but no sperm were found in the smears.

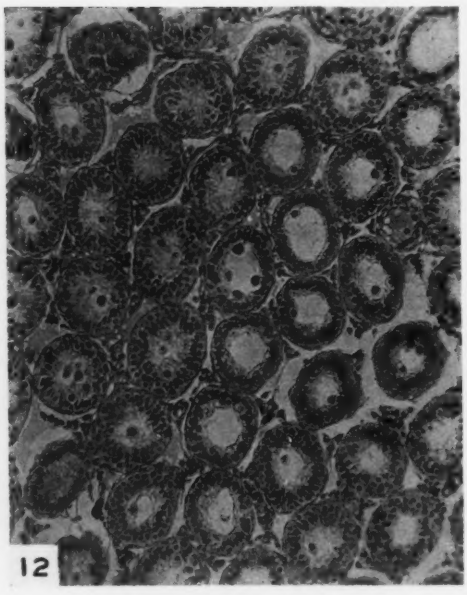
The seminiferous tubules of the thirty-day-old controls contained developmental stages of spermatogenesis no further advanced than spermatids (fig. 4 and 11). Histological study of the testes of the hypophysectomized controls showed a predominance of spermatocytes, though spermatids were found in some tubules (fig. 12). The injection of 1.0 mg. of testosterone propionate per day for thirty days had a variable effect on spermatogenesis. The testes of one of the ten rats with the smallest testes (0.277% of body weight) showed degeneration of the tubules to spermatogonia and a few spermatocytes. Four other rats with testes ranging from 0.366% to 0.500% of body weight possessed spermatids in the tubules and the picture was similar to that of the thirty-day-old controls. Five of the ten rats had testes weighing from 0.564% to 0.657% of body weight and these contained a few tubules with metamorphosing spermatids and sperm heads (fig. 13).

Pellet Series

Absorption of Pellets

The amount of testosterone absorbed from the intratesticular implants of pellets during the thirty-day period can be determined from





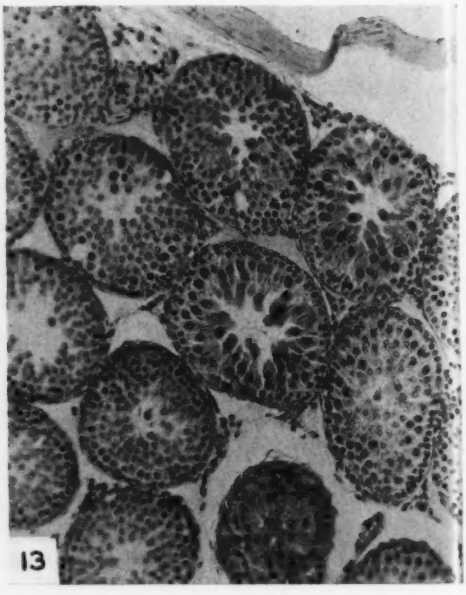


PLATE 3

FIG. 11. Right testis removed at time of hypophysectomy from thirty-day-old rat subsequently injected for thirty days with 1.0 mg. of testosterone propionate per day. Spermatids predominate in the tubules. Weight of testes (right testis ×2), 880.0 mg.

FIG. 12. Testis of rat hypophysectomized at thirty days of age and sacrificed at sixty days of age. Note profound degeneration of tubules to spermatogonia and spermatocytes and the decreased tubule size. Weight of testes, 142.0 mg.

FIG. 13. Left testis of rat hypophysectomized at thirty days of age and injected daily for thirty days with 1.0 mg. of testosterone propionate. Compare with right testis of same rat removed at thirty days of age (figure 11) and note presence of sperm heads in some of the tubules and maintenance of tubule size present at thirty days. Weight of testes (left testis ×2), 591.2 mg.

the size of the pellets recovered from some of the rats at autopsy. Pellets of 100% testosterone, weighing 6.0 to 7.5 mg. were almost completely absorbed in thirty days. The average daily loss in weight of the pellet was 0.20–0.25 mg. and the added injection of 0.1 mg. of testosterone propionate increased the dose of androgen to 0.3 mg. per day in those rats subjected to the combined treatment.

The actual content of testosterone in the 15% pellets weighing 11.2 mg. was 1.68 mg. and the pellets lost 0.8 mg. in weight during the thirty days. If the 0.8 mg. was entirely testosterone, the average daily absorption rate was approximately 0.027 mg. The 5% pellets, weighing 11.0 mg., contained 0.55 mg. of testosterone. No measurable decrease in size of these pellets occurred, but if they had lost half of the volume of testosterone as did the 15% pellets, the average daily dose would be no more than 0.009 mg. per day. These concentrations of pellets probably made little significant contribution to the circulating androgen.

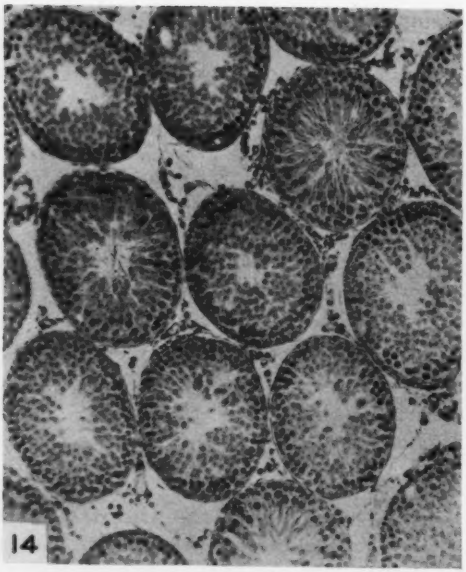
Prostate Weight

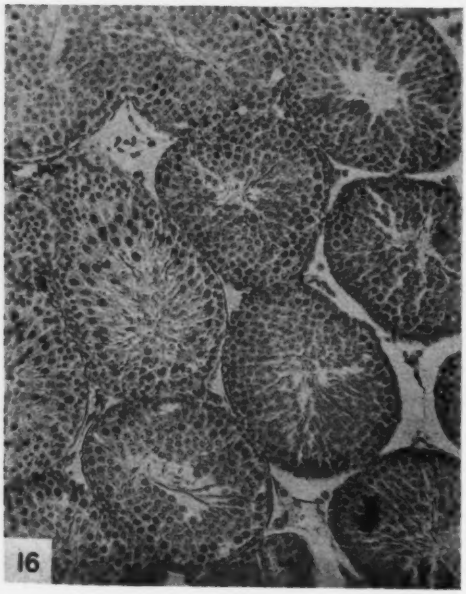
The weight of the prostate gives some indication of the amount of male sex hormone circulating in the body and where intratesticular implants of testosterone pellets served as the sole source of exogenous androgen, this organ may serve as an index of the quantity of testosterone which was absorbed (table 6). The prostate was enlarged with 100% testosterone pellets to twice the size of that of normal sixty-day-old controls, and the addition of 0.1 mg. of testosterone propionate by injection did not further increase the weight. Both the

TABLE 6. DATA CONCERNING THIRTY-DAY-OLD RATS RECEIVING IMPLANTS OF TESTOSTERONE PELLETS IN THE LEFT TESTIS AND DAILY INJECTIONS OF 0.1 MG. TESTOSTERONE PROPIONATE FOR THIRTY DAYS, CONTROLS RECEIVING PELLETS ONLY, AND UNTREATED CONTROLS OF THE SAME AGE

Series	No. of rats	Initial wt. of pellet mg.	Final body wt. grams	Prostate mg.	Testis weight		% difference from right testis	Epididymis weight	
					Right mg. (% body wt.)	Left mg. (% body wt.)		right mg.	left mg.
100% pellets	3	7.5	206	372.4	644.1 (.312 ± .006)	683.9 (.332 ± .007)	6.41%	239.7	353.4
100% pellets and 0.1 mg. t. prop.	6	6.0	177	268.9	598.2 (.339 ± .014)	719.8 (.410 ± .011)	20.94%	227.6	235.5
15% pellets	5	11.2	174	132.6	835.7 (.488 ± .024)	861.4 (.498 ± .020)	2.05%	155.2	164.4
15% pellets and 0.1 mg. t. prop.	9	11.2	212	252.4	299.5 (.142 ± .007)	541.0 (.257 ± .009)	80.99%*	180.5	229.0
5% pellets	4	11.0	197	146.1	1084.0 (.561 ± .026)	902.2 (.462 ± .032)	−17.65%	160.5	163.2
5% pellets and 0.1 mg. t. prop.	6	11.0	215	233.5	350.9 (.164 ± .008)	475.7 (.223 ± .008)	35.98%*	171.3	189.9
60 day Controls	12		214	140.8	1218.1 (.571 ± .009)	1259.6 (.590 ± .009)	3.33%	247.7	242.1
0.1 mg. t. prop.	39		204	210.9	438.4[1] (.209 ± .010)			125.5[1]	

* Statistically significant.
[1] Testis and epididymis weight for this series divided by two.





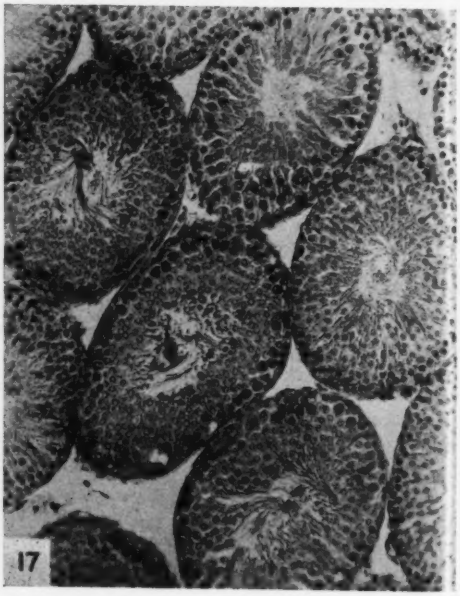


PLATE 4

pellet alone and the pellet plus the injections of testosterone propionate raised the prostate weight above that observed when the treatment consisted of 0.1 mg. of testosterone propionate only. The testosterone from the pellet was obviously reaching the systemic circulation and thus exerting an effect on the contralateral testis.

In the case of the implantation of 15% and 5% pellets, the prostate weights were substantially the same as those of the control rats and the injection of 0.1 mg. of testosterone propionate served only to increase the weight of the gland to the level of that secured in rats injected with 0.1 mg. of testosterone propionate. The amount of androgen supplied by these low concentrations of pellets was not sufficient to stimulate the prostate and in all probability was likewise not concerned in the state of spermatogenesis of the right testis.

Effect on Testis Weight and Spermatogenesis

The implantation of 100%, 15% and 5% testosterone pellets into the left testis of thirty-day-old rats for thirty days did not significantly alter the weight of that testis in comparison with the right testis (table 6, fig. 3). However, a reduction in weight occurred in both testes compared to that of normal sixty-day-old controls. The left testis, nevertheless, showed a greater number of tubules with mature sperm when it contained a 100% testosterone pellet than did the right testis (fig. 14 and 15), and there were more sperm in its epididymis than in the right epididymis; the left epididymis was also heavier than the right.

No difference in the histological picture between the two testes was found with 15% and 5% pellet implants; both testes were normal in appearance. The left epididymis in the 15% pellet group, though not appreciably heavier than the right, contained more sperm. The epididymis weight and sperm content on the two sides were similar in the 5% series (table 6).

When 0.1 mg. of testosterone propionate was injected per day during the thirty days, significant increases in the left testis weight were found with implantations of 15% and 5% pellets (80.99% and

FIG. 14. Right testis of thirty-day-old rat receiving implant of 100% testosterone pellet in left testis and sacrificed at sixty days of age. Note inhibition of sperm formation in some tubules and a decrease in tubule size. Right testis weight, 687.2 mg.

FIG. 15. Left testis of same rat containing implant of 100% testosterone pellet for thirty days. Note normal appearance of seminiferous tubules. Left testis weight, 724.6 mg.

FIG. 16. Right testis of thirty-day-old rat receiving implant of 100% testosterone pellet in left testis and injections of 0.1 mg. of testosterone propionate per day for thirty days. Compare with figures 7 and 14 and note larger tubules and better maintenance of spermatogenesis with both pellet implant and injections than with either form of treatment alone. Right testis weight, 529.6 mg.

FIG. 17. Left testis containing 100% testosterone pellet of same rat. Compare with figure 16 and note increase in tubule diameter and numbers of sperm with pellet implant. Left testis weight, 676.0 mg.

35.98% heavier than right testes). The weight of the left testis was increased, but not significantly, with the 100% testosterone pellets. Spermatogenesis was improved in all testes containing pellets. In the case of implants with 100% testosterone pellets, the inhibiting effects of the 0.1 mg. of testosterone propionate were overridden by the additional testosterone supplied by the pellet and even the right testis was increased in weight beyond that secured with injections of 0.1 mg. of testosterone propionate alone, though not as markedly as the left. Spermatogenesis was normal in the left testis and only slightly inhibited in the right testis when the former contained a 100% testosterone pellet (table 6, fig. 16 and 17).

The 15% and 5% pellets were not of sufficient concentration to

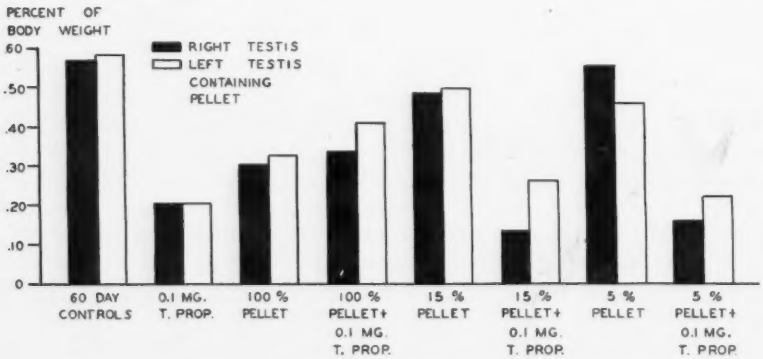
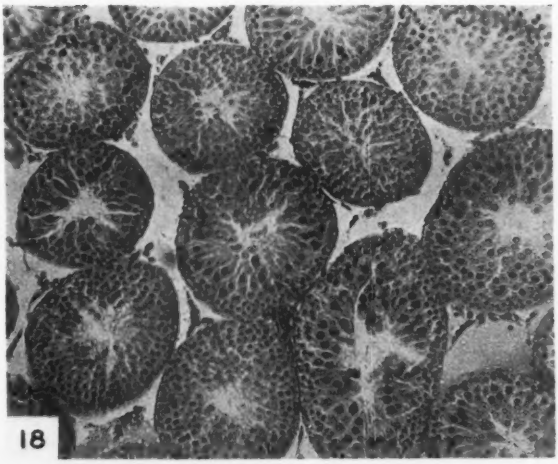


FIG. 3. Graph of testis weight (expressed as per cent of body weight) of thirty-day-old rats receiving intratesticular implants of testosterone pellets over a thirty-day period and of controls of the same age.

affect the right testis and consequently the weight of this testis was not improved by the pellet implant in the left testis in the presence of the injected testosterone propionate and spermatogenesis was halted at the spermatid stage (fig. 18). However, the local effect of the implanted pellet was manifested in the 80.99% increase in left testis weight over that of the right testis with the 15% pellet and the 35.98% increase with the 5% pellet (table 6). In contrast to the uniform picture obtained in all parts of the testes implanted with 100% testosterone pellets, the seminiferous tubules in the testes containing 15% and 5% pellets varied in their development according to their proximity to the pellet. The tubules in the near vicinity of the pellet contained metamorphosing spermatids and mature sperm (fig. 19) while those at the ends of the testis had not matured beyond the stages of spermatocytes and spermatids (fig. 20). There was seen, therefore, a local stimulating effect of the testosterone on the seminiferous tubules which counteracted the inhibiting effect of the injected 0.1 mg. of testosterone propionate.



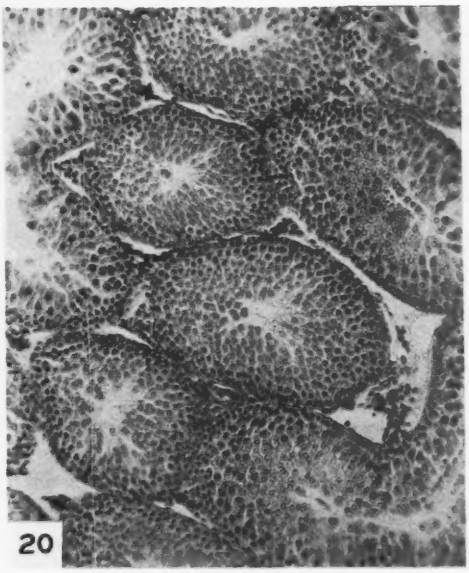


PLATE 5

FIG. 18. Right testis of thirty-day-old rat with 5% testosterone pellet in left testis and injected with 0.1 mg. of testosterone propionate per day for thirty days. Compare with testis shown in figure 7 from 0.1 mg. injected rat. Right testis weight, 336.4 mg.

FIG. 19. Tubules located immediately around 5% testosterone pellet implanted in left testis of same rat. Note increase in tubule diameter compared with that shown in figure 18 and presence of sperm. Left testis weight, 473.2 mg.

FIG. 20. Tubules located in same section as in figure 19 but at the opposite edge of the testis from the pellet implant. Note lack of stimulation of spermatogenesis in these tubules at a distance from the pellet, but increase in size of tubules over that seen in the right testis (figure 18).

The smears of sperm from the epididymis of these two groups confirmed the local stimulating action of the pellets, for in those rats with 15% and 5% testosterone pellets implanted in the left testis and receiving 0.1 mg. of testosterone propionate per day there were no sperm in the right epididymis but the left contained some sperm with poor motility.

The interstitial cells were reduced in size in both testes of the recipients of the 100% testosterone pellets (fig. 14 and 15), but little or no change from the control condition was seen in the interstitial cells of either the right or the left testis with implants of 15% and 5% testosterone pellets. The volume of intertubular tissue was diminished in the testes of the rats which had been treated with 0.1 mg. of testosterone propionate in addition to intratesticular pellet implants (fig. 16–20).

DISCUSSION

General Effects of Androgen Administration

The somatic effects of the administration of androgen to sixty-day-old and thirty-day-old rats were similar in both age groups. The gain in body weight over the period of treatment was not significantly affected in the older rats and a significant decrease in weight gain was achieved in the thirty-day-old series with the heaviest doses of testosterone propionate (2.0 and 3.0 mg. per day) alone. The normal diminution in thymus size which accompanies ageing was hastened by treatment with androgen in all groups receiving 1.0 mg. or more of testosterone propionate. The usual picture of stimulation of the prostate and seminal vesicles by androgen was found in all groups except those receiving the lowest dose (0.01 mg.) in the thirty-day-old series.

In the sixty-day-old treated rats the epididymis weights were not markedly altered from those of the controls except where the sperm content was reduced by the injections (0.1 mg. per day). The variations in weight of the epididymis at the different dosage levels in the thirty-day-old rats were as much correlated with the quantity of sperm present in the duct as with the dose of testosterone propionate administered. Sperm storage is apparently as important as the volume of duct tissue in the weight of the epididymis.

The "compensatory atrophy" of the adrenals described by Selye (1940b) following androgen injections in immature rats from birth to thirty days of age is evidently not secured when the rats are exposed to heavy doses of androgen over the thirty-to-sixty day and the sixty-to-ninety day age intervals, for no significant change in adrenal weight was noted in any of the treated groups. Pituitary weights were not significantly altered by the androgen injections except in those thirty-day-old rats receiving 2.0 and 3.0 mg. of testosterone propionate per day where a slight decrease occurred. This absence of effect of andro-

gen on the weight of the adrenals and pituitary has been previously reported by Korenchevsky, Dennison and Kohn-Speyer (1933) and McEuen, Selye and Collip (1937).

The renotrophic effect of male sex hormone which was first noted by Korenschevsky, Dennison and Kohn-Speyer (1933) was secured in both age groups with significant increases in kidney weight occurring with the larger doses of testosterone propionate.

Estrogen injections are known to inhibit growth (Bugbee and Simond, 1926; Spencer, Gustavson and D'Amour, 1931) and this observation was confirmed in the lesser weight gains of the thirty-day-old rats receiving 4.2 and 8.4 μg. of estradiol per day. The addition of 1.0 mg. of testosterone propionate per day to the estradiol injections permitted a more normal gain in weight over the period of treatment.

The remarkable increase in adrenal weight secured with estrogen treatment (63% with 4.2 μg. of estradiol and 90% with 8.4 μg.) in the thirty-day-old series, and the suppression of this hypertrophy with the simultaneous administration of 1.0 mg. of androgen are similar to the results reported by Selye (1940a). The pituitary hypertrophy which results from estrogen treatment is well-known and the prevention of this increase in weight by the addition of androgen to the estrogen treatment, observed by Wolfe and Hamilton (1937) and Selye (1940a) was present to an even greater degree in this experiment, a 14% decrease in weight occurring when 8.4 μg. of estradiol and 1.0 mg. of testosterone propionate were injected each day.

The weight changes in the pituitary with estrogen and androgen treatment are interesting in the light of the assays of their gonadotrophic potency. The significant decrease in weight noted with the heavy doses of testosterone propionate are associated with low gonad-stimulating ability whereas the increase with estradiol treatment likewise accompanies diminished potency.

Estradiol had a slight inhibiting effect on thymus weight at the dosage levels used in this experiment. Kidney weights were not greatly affected but the trend was toward a lower weight in the treated animals than in the controls, an effect reported previously by Selye (1940a).

Pituitary Assays

The decline in gonadotrophic potency of pituitaries dried in acetone was such that three times as many glands were needed to produce stimulation of the ovaries of immature female rats comparable to that secured with one fresh frozen pituitary. Further loss of potency occurred with delay between sacrifice of the male rats and the assay of the dried pituitaries, and since a supply of immature female rats was not always available the results from these assays are less reliable than from assays using frozen suspensions. The gradual reduction of gonadotrophin content secured with increased dosage of testosterone pro-

pionate in the sixty-day-old series would undoubtedly have been even more evident with the use of fresh frozen pituitaries.

Ovarian weight of immature female rats was chosen in preference to uterine weight as the criterion for determining gonadotrophic potency of the pituitaries because the weight of the ovaries was in direct relation to the effect of the gonadotrophins on the follicles. Pituitaries containing small amounts of gonadotrophins caused follicular growth and a slight increase in weight whereas greater pituitary stimulation produced heavier ovaries due to the formation of corpora lutea. Uterine weight, dependent on ovarian hormone production, was greatest during the ovarian follicular phase when the uteri were filled with fluid and declined with the loss of this fluid following luteinization of the follicles.

Effects of Androgen Administration on the Testis

The apparently anomalous effect of androgen on the testis of the rat reported in the literature, namely, an injurious effect resulting in lowered testis weight and cessation of spermatogenesis in the intact animal and a stimulating effect in the hypophysectomized rat whereby testis weight and formation of sperm are maintained, can be resolved into a reasonable explanation when the various factors responsible for the maintenance of testis function are considered.

The importance of the anterior pituitary hormones to normal growth and maturation of the testis has been well demonstrated and any experimental procedure which results in a decrease in the supply of the gonadotrophic secretion causes an inhibition of the testis. Hypophysectomy obviously removes the source of this normal gonad-stimulating factor, the absence of which is responsible for the extreme gonadal degeneration which follows. Injections of female sex hormone suppress the pituitary and reduce the output of gonadotrophins so that the seminiferous tubules and Leydig cells regress to a state comparable to that found following hypophysectomy. Similar suppression of the pituitary is found when male sex hormone is administered, but unlike estrogen, androgen exerts a dual effect on the testis which stimulates the seminiferous tubules directly when the threshold is passed at which its action is only effective as an inhibitor of the pituitary. Thus a stimulating effect both on testicular weight and maturation of germinal elements is obtained when androgen is administered in sufficiently large doses to hypophysectomized rats; the same stimulation is secured with combined estrogen and androgen injections in intact rats and in rats treated with large doses of male sex hormone alone. All doses of androgen suppress the pituitary in proportion to their size, but with large doses the inhibiting effect on the pituitary and thence on the testis is negated by the concomitant stimulating effect of the androgen on the seminiferous tubules.

In the thirty-day-old rat the testis is still immature and the process

of spermatogenesis has not proceeded beyond the spermatocyte and spermatid stages. In the course of the next ten days sperm begin to appear in the tubules and by sixty days the gonads are essentially mature. Administration of testosterone propionate over this age interval will either permit spermatogenesis to progress to completion or will interrupt it at some intermediate stage. With doses of 0.01 to 0.04 mg. of testosterone propionate the inhibition of the pituitary is slight and the 20% decrease in testis weight is the only evidence of testicular inhibition. With the injection of 0.1 mg. of testosterone propionate the decline in gonadotrophic potency reaches a crucial level at which a 65% decrease in the weight of the testes occurs and the process of spermatogenesis does not proceed beyond its immature thirty-day-old state. With increases in dosage to 1.0, 2.0 and 3.0 mg. per day the pituitary gonadotrophic potency continues to decline, but the level of androgen in the blood is raised to a point where it can act directly on the seminiferous tubules to permit the formation of sperm.

Administration of 8.4 μg. of estradiol depletes the pituitary of its gonadotrophic potency, the weight of the testes is extremely reduced, and the most advanced stages of spermatogenesis to be found are spermatogonia and spermatocytes. No significant change occurs in gonadotrophic potency with simultaneous administration of 1.0 mg. of testosterone propionate, but testicular weights in these animals return to near normal levels and spermatogenesis is stimulated.

In half of the rats hypophysectomized at the age of thirty days in this experiment and treated for thirty days with 1.0 mg. of testosterone propionate, sperm heads were found in some of the seminiferous tubules and it is probable that 3.0 mg. of testosterone propionate would have increased the incidence and volume of sperm in rats hypophysectomized at this age. Thus, in the total absence of the pituitary hormones in a thirty-day-old rat, stimulation of the testes to form mature sperm is possible with male sex hormone.

Less damage to the testis following testosterone propionate administration is seen in the sixty-day-old series than in the younger rats. The 24% decrease in weight with the 0.1 mg. dose of testosterone propionate is much less than that found with similar dosage in the thirty-day-old series (65%) and, as far as the histological picture is concerned in the sixty-day-old rats, the process of spermatogenesis is not affected. Moore and Price (1938) have suggested that the pituitary is less easily modified by androgen treatment in the adult animal than in the immature, but the results of this experiment show a marked depletion of pituitary gonadotrophic potency with all doses of testosterone propionate in the sixty-day-old series and it appears that the mature testis is less sensitive to lowered gonadotrophin levels rather than that the pituitary is less affected by the androgen. The increased testis weight with the heavier doses of testosterone propionate (1.0 and 3.0 mg. per day) is evidence that the testis is being stimulated

directly by the androgen in spite of the lowered gonadotrophic potency of the pituitary.

Further evidence of the gonadotrophic effect of male sex hormone is seen in the series of thirty-day-old rats with intratesticular implants of testosterone pellets, receiving injections of 0.1 mg. of testosterone propionate per day for thirty days. This dose of testosterone propionate was sufficient to suppress the pituitary gonadotrophin secretion but not to stimulate the testes directly. With implants of 100% testosterone pellets in the left testis, spermatogenesis was better maintained in this testis than in the right, but both testes contained sperm. With the 15% and 5% pellets in rats treated with 0.1 mg. of testosterone propionate per day the right testes were inhibited to the same extent as with 0.1 mg. alone, but sperm were to be found in the left testis in the immediate vicinity of the pellets. The amount of testosterone absorbed from these pellets was so small that only those tubules in close proximity to the pellet were exposed to a sufficient concentration of the diffused hormone to permit the maintenance of spermatogenesis. Similar results with low concentrations of pellets have been obtained in the hypophysectomized rat by Dvoskin (1944).

Whether this gonadotrophic action of the male sex hormone is involved in the initiation and maintenance of spermatogenesis in the intact animal is not known. The first suggestion that such a mechanism might be operative in pituitary-gonad relationships came with the observations of various workers that the luteinizing hormone found in pregnancy urine (chorionic gonadotrophin) would maintain spermatogenesis in the hypophysectomized rat as well as the accessory glands through stimulation of the interstitial tissue (Smith and Leonard, 1933). Purification of an "interstitial cell stimulating hormone" (ICSH, presumably identical with LH) from pituitary extracts provided Simpson, Li and Evans (1942) with further evidence of the ability of this luteinizing hormone to maintain spermatogenesis following hypophysectomy. Hamilton and Leonard (1938) compared the action of male sex hormone in maintaining testis function in the hypophysectomized animal with the effects of pregnancy urine extract and surmised that the stimulating effect of the chorionic gonadotrophin on the interstitial cells caused the secretion of androgen which in turn supported spermatogenesis. That the maintenance of spermatogenesis in hypophysectomized androgen-treated rats was probably due to a direct action of androgen on the seminiferous epithelium and not as a result of maintenance of a normal scrotum was shown by Nelson (1937).

Simpson, Li and Evans (1944) and Simpson and Evans(1946) found that doses of pregnancy urine, pregnant mare serum and ICSH would cause production of sperm in rats hypophysectomized at forty days of age when administered in doses too small to maintain the interstitial tissue of the testis and the accessory sex organs, and they

questioned whether the gonadotrophic agent caused the interstitial cells to produce a steroid more effective in tubule stimulation and less effective in stimulation of the accessory organs of the male than the synthetic androgens. The absence of direct correlation between the capacity of an androgen to maintain spermatogenesis and its capacity to stimulate accessory sexual organs had been demonstrated by Nelson (1937) for a variety of synthetic androgens. The fact that the spermatogenic process attains its final stages in pubertal animals at a time when the accessory organs are relatively infantile further emphasizes the probability that any part played by androgens or related steroids in the process is not related directly to their capacity to stimulate the accessory reproductive organs.

However, exogenous androgen has not been able to induce sperm formation in rats hypophysectomized before the 29th day of age according to Cutuly and Cutuly (1940). They have explained this deficiency as a necessity for the presence of the hypophyseal gonadotrophins for the meiotic divisions in the process of spermatogenesis whereas succeeding mitotic stages, including sperm formation, can occur in the absence of the pituitary under the influence of the male hormone. This explanation can scarcely be regarded as entirely satisfactory since meiotic activity can be observed in the testes after hypophysectomy and, furthermore, it has been shown that spermatogenesis can be maintained with androgen for at least 178 days after hypophysectomy (Nelson, 1940b). It has also been possible to reinstate the final stages of the spermatogenic process with androgen in adult rats which were hypophysectomized at least twenty-eight days prior to treatment (Nelson, 1940a).

The male sex hormone is apparently not a complete substitute for pituitary gonadotrophins for even with heavy doses of testosterone propionate in this present experiment, the testes of the rats were subnormal in weight. The additional evidence secured by other investigators from the treatment of hypophysectomized rats with androgen in which testicular weight could not be elevated above that which prevailed at the time of hypophysectomy also attests to the need for pituitary gonadotrophins for full gonadal activity. Gaarenstroom and deJongh (1946) have explained this latter situation in terms of a special action for the pituitary gonadotrophin affecting the germinal epithelium by which it increases the number of immature germ cells with the potentiality for division and thus enlarges the tubules, an ability not given to androgens which act only to maintain those dividing spermatocytes present at the time of hypophysectomy.

It is quite true that the seminiferous tubules in the testes of androgen-treated hypophysectomized males are smaller than normal and it is likely that this accounts for a major share of the failure of such testes to maintain normal weights although very active spermatogenesis may be in progress. A second, and probably minor, factor

which contributes to the lower testicular weights is the atrophic condition of the intertubular tissue. Although Gaarenstroom and deJongh offer insufficient evidence to prove their thesis, their suggestion that hypophyseal gonadotrophic hormone is necessary for normal maintenance of spermatogonial divisions may be a satisfactory hypothesis to explain the smaller seminiferous tubules of androgen-treated hypophysectomized rats.

SUMMARY AND CONCLUSIONS

Injections of testosterone propionate in doses of from 0.01 to 3.0 mg. per day in thirty-day-old rats and from 0.05 to 3.0 mg. per day in sixty-day-old rats over a thirty-day period resulted in a significant reduction in testicular weight with all doses administered to the younger rats and with injections of 0.1 and 1.0 mg. in the older series. The greatest dimunition in the weight of the testes in both age groups occurred with injections of 0.1 mg. of testosterone propionate.

No obvious histological evidence of spermatogenic inhibition, other than a slight decrease in tubule size, was found in any of the testes except those of the thirty-day-old rats receiving 0.1 mg. of testosterone propionate per day where suppression of spermatogenesis to spermatocyte and spermatid states accompanied the severe inhibition of testicular growth. Decreased volume of sperm in the epididymis was associated with the lowered testicular weight secured with doses of 0.01 and 0.1 mg. in the thirty-day-old series and with 0.1 mg. in the sixty-day-old rats. The decrease in numbers of sperm is evidence that the rate of sperm production has been reduced although the latter may not be readily detected by examination of the tubules.

Daily injections of 4.2 and 8.4 μg. of estradiol for thirty days caused an even greater suppression of testicular growth and spermatogenesis than that secured with 0.1 mg. of testosterone propionate per day in thirty-day-old rats. Simultaneous administration of 1.0 mg. testosterone propionate prevented this damage from the estradiol injections. Doses of 1.0 mg. of testosterone propionate per day likewise prevented some of the testicular weight loss following hypophysectomy of the thirty-day-old rat and maintained spermatogenesis at spermatid and sperm head stages.

The injurious effect on the testis of injections of 0.1 mg. of testosterone propionate per day was prevented when pellets of testosterone were implanted in one testis. Spermatogenesis was maintained in both testes with 100% testosterone pellets but only in the testis containing the pellet with 15% and 5% concentrations and there in an area limited to that immediately surrounding the implanted pellet.

Assay of the pituitaries of the treated rats in immature female rats showed a decreased content of gonadotrophin with both androgen and estrogen injections, ranging from slight reduction with low doses to complete depletion with the heaviest doses. However, testicular

weight was maintained at near normal levels with the heavier doses of testosterone propionate (1.0, 2.0 and 3.0 mg. per day) in spite of the extreme reduction in gonadotrophic potency of the pituitaries with these injections. The greatest inhibition of testis function occurred with injections of 0.1 mg. of testosterone propionate, a dose which did not completely suppress the pituitary.

From the data presented in this experiment, the conclusion is reached that low doses of testosterone propionate suppress the secretion of gonadotrophins by the pituitary and thus indirectly injure the testis, producing loss of weight and inhibition of sperm formation. High doses, while they likewise inhibit the pituitary, result in a level of androgen which stimulates the seminiferous tubules directly. Thus testicular weight is maintained and spermatogenesis proceeds in a normal manner even in the face of a diminished supply of gonadotrophic hormones.

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THE ADDITION OF EGGS TO THE HEN'S CLUTCH BY REPEATED INJECTIONS OF OVULATION-INDUCING HORMONES

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THE HEN usually lays her eggs at the rate of one a day for several days, misses a day, and then approximately repeats this performance. Her clutch may vary in length from 1 egg to more than 100, but generally it contains fewer than 4 or 5 eggs. Ovulation does not occur on the day the terminal egg of the clutch is laid, but some 14 to 18 hours later, or early in the morning of the following day (Warren and Scott, 1935).

Previous work has shown that ovulation can be induced in the normal hen by anterior pituitary gonadotrophin (Fraps, Olsen, and Neher, 1942) and by progesterone (Fraps and Dury, 1943). In regularly laying hens the first follicle of an oncoming clutch can be caused to ovulate readily by as much as 24 hours' prematurity, and, by properly timed injections, oviposition of the terminal egg of one clutch and ovulation of the first follicle of a succeeding clutch approximate the relation characteristic of normal intraclutch successions (Fraps, 1942). Thus a normal 2-egg clutch became a 3-egg clutch, the normal 3-egg clutch became a 4-egg clutch and so on.

The purpose of the present study was to induce repeated ovulations in hens of low or moderate production by adding a first ovulation at or near the end of a 2-egg clutch and, by repeating injections of appropriate hormones at 24, 26 or 28 hourly intervals, to force ovulations until the ovary failed to respond.

MATERIALS AND METHODS

Most of the hens used in the experiments described here were of the White Leghorn and Rhode Island Red breeds, but some crossbred, New Hampshire and Dark Cornish birds were included. The hens were kept in laying batteries, with access to feed and water at all times, and were subjected to artificial lighting from 6:00 A.M. until 8:00 P.M. As a routine procedure in this laboratory, eggs laid were recorded at 6:00 A.M., hourly from 8:00 A.M. through 4:00 P.M., and again at 8:00 P.M. The number of eggs any hen might be expected to lay in a forthcoming clutch could be predicted with a reasonable degree of accuracy on the basis of these routine records of prior performance.

Ovulations were induced by the subcutaneous injection either of an air-dried unfractionated preparation of anterior pituitary glands from male fowl, or of progesterone. The anterior pituitary tissue was suspended in

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distilled water immediately before injection, 2.0 mg. tissue per ml. water; injections were made subcutaneously rather than intravenously in order to avoid possible damage to veins as a result of repeated injections. Progesterone was dissolved in corn oil, 10 mg. per ml. for injections at 0.6 and 0.9 mg. levels, 1 mg. per ml. for injection at the 0.3 mg. level.

The second egg of the normally ovulated 2-egg clutch and its yolk were weighed, as controls, for comparison with weights of eggs and yolks resulting from forced ovulations. None of the injected hens were autopsied, and it is therefore not possible to estimate the extent to which yolk deposition, as measured in terms of yolk-bearing follicles on the ovary, may have occurred during the course of successively forced ovulations.

Timing of injections. Under conditions prevailing in this laboratory, the first egg of a 2-egg clutch is usually laid between 9:00 A.M. and 12:00 noon. Ovulation of the second ovum of the normal 2-egg sequence usually occurs within an hour thereafter (Phillips and Warren, 1937; Neher, Olsen and Fraps, in press). The egg formed consequent to this terminal ovulation of the 2-egg clutch is laid during early afternoon hours of the following day, ordinarily between 1:00 and 4:00 P.M.

In all tests reported in this paper, the first injection of ovulation-inducing hormone was timed to bring about ovulation of the first follicle of an oncoming clutch at approximately the time of lay of the second (or terminal) egg of a normal 2-egg clutch, that is, between approximately 1:00 and 4:00 P.M. of the day following ovulation of this second ovum. Injections were accordingly made between 4:00 and 8:00 A.M. of the day following ovulation of the second ovum on the supposition that ovulation would be forced some 6 to 8 hours following injection (Fraps, Riley, and Olsen, 1942;* Fraps and Dury, 1943). Each hen was palpated at time of first injection for presence or absence of hard-shelled egg *in utero*, and only hens carrying hard-shelled eggs were injected for forced ovulation.

Injections subsequent to the first were made at 24, 26 or 28 hour intervals to force ovulations at intervals corresponding approximately with those of normal intra-clutch ovulatory sequences. Each hen was palpated before injection, and hens failing to respond to the first or any subsequent injection, as indicated by absence of uterine eggs, were given no further treatment.

Lay of forced eggs. The time of lay of the 2 eggs constituting the normal 2-egg clutch was known from the routine recordings noted previously. Following injections for forced ovulations, hens of the several experimental groups were observed at hourly intervals, beginning usually somewhat before the lapse of 24, 26 or 28 hours following lay of the previous egg, in consonance with corresponding injection schedules. A few hens laid their eggs before observations were begun, but all others were followed until the uterine egg was laid.

Controls. It was a matter of prime importance to know that clutches of more than 2 eggs would not be laid by any considerable number of hens dur-

* The interval given by these authors was based on intravenously injected gonadotrophin. All injections were made subcutaneously in the tests described here, and it is possible that the interval of 6-8 hours is not entirely accurate for administration of pituitary gonadotrophin by this route. It should also be noted that much higher quantities must be injected to force ovulation by the subcutaneous route.

ing the course of injections, since the addition of eggs to the normally expected clutch was predicated upon the assumption that ovulations following injections were caused by the injected hormone, and would not have occurred consecutively in the absence of hormonal administration. Sixty hens were accordingly selected on exactly the same basis as were the tested hens. Four of these laid 3-egg clutches instead of the expected 2-egg clutches; none laid clutches of more than 3 eggs. Of the 53 hens subjected to experimental procedures, it thus seems unlikely that more than 4 or 5 might have normally

TABLE 1. RECORDS OF LAY OF HENS PRECEDING, DURING THE COURSE OF, AND FOLLOWING ADMINISTRATION OF 0.60 MG. PROGESTERONE DAILY AT 8:00 A.M.

Hen	Lay preceding treatment											Lay during and following treatment											Eggs added
1755	–	e	–	e	–	e	–	e	e	–	e	e	e	–	–	–	e	e	–	e	–	e	e	2
2629	–	e	e	–	e	e	–	e	e	–	e	e	–	e	–	–	e*							1
3967	–	e	–	e	e	–	e	–	e	–	e	e	e	–	–	–	–	–	e	e	–	e	e	2
7102	–	–	e	–	e	e	–	e	–	–	e	e	e	–	–	–	–	–	–	–	–	–	–	2
7115	–	e	e	–	e	e	–	e	e	–	e	e	–	–	–	–	–	–	–	–	–	–	–	1
7534	–	e	e	–	e	e	–	e	e	–	e	e	e	–	–	–	–	–	e	–	e	–	e	2
8021	e	e	–	e	–	e	–	e	e	–	e	e	e	e	e	e	e	–	e	e	–	e	e	–	5
8747	–	e	e	–	e	e	–	e	e	–	e	e	–	e	e	e	–	–	–	–	–	–	e	–	4
9792	e	–	e	e	e	–	–	e	e	–	e	e	e	e	–	–	e	–	–	e	–	e	e	3
9927	–	e	e	–	e	e	–	e	e	–	e	e	e	e	e	e	–	–	–	–	e	e	–	e	4

* Hen removed from cage.
Lay on a given day is indicated by the letter e, which is underscored for days on which injections were made. The dash signifies a day of missed lay.

laid 3-egg clutches, a proportion which would not have altered materially the recorded findings. Three hens of group F (table 2) are noted specifically in connection with the results for that group.

RESULTS

Records of lay of individual hens before, during, and following injection of progesterone, 0.6 mg. per hen at 24-hour intervals, are given in table 1 (these constitute group D of table 2). The recorded results are fairly representative of those of hens in the other five groups, except one hen of group F, in which 13 ovulations were forced, and a few hens which failed to respond by the addition of even a single ovulation to the normally expected 2-egg clutch.

It will be noted that only one hen (9792) of table 1 laid 3 eggs successively within the 12 days preceding initial injections. Five of the 10 hens regularly laid 2 eggs on successive days followed by a day of missed lay; in the other five birds (including hen 9792) one or more single-egg clutches was interposed within the 12-day period. The greatest number of eggs added to the expected 2-egg clutch was 5 (hen 8021), the least was 1 (hens 2629 and 7115). Average additions for the group were 2.5 eggs.

Following failure of ovulation to occur in consequence of injection, only one hen, 8021 of table 1, to whose expected clutch of 2 eggs 5 were added, resumed her characteristic schedule with but a single day's break between clutches. Lapses following forced ovulation and lay ranged from 2 to 11 of the 12 days of record covered by table 1.

Excluding hen 2629 (removed from test), the remaining 9 hens of the group laid 68 eggs during the 12 days before initial injections, 49 eggs during the 12 days beginning with lay of the first forced egg.

The results of all tests with male fowl anterior pituitary tissue and with progesterone are summarized in table 2. The average number of eggs added to clutches of hens of group A was only a little less

TABLE 2. EGGS ADDED TO ANTICIPATED 2-EGG CLUTCHES FOLLOWING INJECTION OF MALE CHICKEN ANTERIOR PITUITARY TISSUE (AP) AND PROGESTERONE (PG.)

Group	Hens	Injection			Eggs added	
		Hormone	Quantity	Interval	Per hen	Range
	No.		mg.	hrs.	No.	No.
A	10	AP	1.0	24	2.4	0–5
B	6	AP	1.0	26	3.0	1–5
C	10	Pg.	0.3	24	2.0	0–5
D	10	Pg.	0.6	24	2.5	1–5
E	10	Pg.	0.9	24	2.5	1–4
F*	7*	Pg.	0.3–0.9*	28	4.7 (3.3)†	1–13 (1–7)†

* The birds in this group received 2 injections of 0.3 mg., 2 of 0.6 mg., and all subsequent of 0.9 mg. progesterone.
† Except hen 2477; see text.

than in group B, although the interval between successive injections was longer for hens of group B (26 hours as against 24 hours in group A).

Progesterone at the 0.3 mg. level yielded a somewhat lower average number of eggs added than at the 0.6 or 0.9 mg. level (groups C, D, and E respectively, table 2), but the maximal number of eggs added was 5 for the lower injection levels (groups C and D) as against 4 for the groups at the higher level. All hens of groups C, D, and E were injected at 24-hour intervals, and are in this respect comparable with group A.

Including the record of hen 2477, which added 13 eggs to her anticipated 2-egg clutch, the mean for group F was 4.7 eggs added; excluding this hen, the mean was 3.3 eggs, range 1–7. The results from this group are perhaps not directly comparable with those from preceding groups, since 3 of the 7 hens were removed from outside flocks during February and were confined to laying cages less than 21 days prior to first injection, hen 2477 for only 12 days. These three hens were thus subjected to a lengthened light period at a time of the year when increased lighting is commonly followed by increased production (and clutch length). A single ovulation was added to the normal 2-egg sequence in one hen, 7 in the second and 13 in the third. Moreover, progesterone was injected at graduated levels and at a greater interval between successive injections than in other tests.

The record of hen 2477 is of particular interest in view of her continued response to 13 repeated injections. Approximate times of lay

by this hen, together with egg and yolk weights for the second egg of the anticipated 2-egg clutch and the 13 added eggs are given in table 3. Decline in weights of her eggs and contained yolks was appreciable only following lay of the fifth added egg and continued with fair regularity thereafter. The final yolk of the series weighed only 10.1 grams, in contrast with 15.7 grams for the second yolk of the normal 2-egg clutch.

TABLE 3. EGG AND YOLK WEIGHTS OF THE SECOND EGG OF A NORMAL CLUTCH AND OF 13 SUCCESSIVE, EXPERIMENTALLY ADDED EGGS (HEN 2477)

Approximate time of lay	Time elapsed from preceding lay	Egg weight	Yolk weight
	hrs.	gms.	gms.
12:00 A.M.	—	55.0	15.7
2:00 P.M.	26	53.4	14.8
6:00 P.M.	28	54.5	14.5
10:00 P.M.	28	55.4	14.2
3:00 A.M.	29	56.2	14.7
7:00 A.M.	28	56.0	14.7
11:00 A.M.	28	53.5	14.0
3:00 P.M.	28	52.0	13.3
7:00 P.M.	28	51.4	13.3
12:00 P.M.	29	50.6	12.4
5:00 A.M.	29	50.6	12.3
9:00 A.M.	28	50.6	12.3
12:00 A.M.	27	47.9	11.3
4:00 P.M.	28	44.4	10.1

Three hens in group E and one in group F held eggs arising from forced ovulations for 1 to 4 days beyond time of expected lay. All these hens received progesterone at the 0.9 mg. level. A similar retention of uterine eggs was noted by Dunham and Riddle (1942) in the pigeon following the injection of progesterone, and, in this laboratory, in the hen following the administration of progesterone adsorbed on carbon black (unpublished).

DISCUSSION

Although this paper has dealt with the addition of eggs to the hen's clutch, the typical clutch of the regularly laying hen was in fact completely obliterated in many instances. Under conditions maintained in this laboratory, all eggs of a given clutch, regardless of clutch length, are normally laid within daylight hours, but as table 3 shows, eggs resulting from injections at 28-hour intervals were laid at fairly corresponding intervals completely around the clock, and presumably ovulations were likewise spaced. A similar extension of normal clutch bounds occurred in all the longer sequences when injections were made at 26- or 28-hour intervals, successive eggs being laid (and successive ovulations being induced) approximately 2 or 4 hours later each day. The observation that the injected hens laid and ovulated during hours of darkness is not in itself notable, for Warren and Scott (1936) found the same to be true, for a time, in hens subjected to reversal of lighting. What seems of more significance is that

the normal environmental controls over ovulation and lay can apparently be completely over-ridden so long as the enforced sequence of ovulations remains unbroken.

It is clear from table 2 that the results of injections were highly variable (see range for each group). Results are summarized in somewhat different form in table 4. The 3 hens failing to respond by even a single ovulation were in all probability due normally to miss two days rather than a single day between clutches, although the possibility of

TABLE 4. HENS GROUPED BY NUMBER OF OVULATIONS INDUCED PER HEN*

Hens	Ovulations per hen
No.	No.
3	0
13	1
10	2
11	3
7	4
6	5

* Excluding the 3 lately-caged hens.

inadequate dosages (for these particular hens) cannot be ruled out. Any of several factors may have accounted for termination of the remaining sequences, the most probable being lack of mature or ovulable ovarian follicles at the time of failure of ovulation. The fact that results were of the same order with pituitary gonadotrophin, which apparently acts directly on the follicle, and progesterone, which effects ovulation by causing release of ovulation-inducing hormone from anterior pituitary gland (Rothchild and Fraps, 1949), would support this conclusion. Particularly in the longer sequences, weights of successively ovulated yolks declined steadily; for the 13 hens in which ovulation was forced at least 4 times (last 2 groups of table 4), mean weights of the second normally ovulated ova and of the 4 succeeding forced ova were 17.4, 16.8, 16.2 15.5, and 14.3 grams, the mean difference between weights of the first and last ova of the series being 3.1 grams. An even more marked decline in weights of successive ova, beginning notably with the sixth forced ovulation, occurred in hen 2477 (table 3). The inference is that the hen's pituitary gland failed to secrete sufficient follicle-stimulating hormone to bring follicles to maturity as rapidly as they were ovulated.

Although yolk size declined to sub-normal values in the longer injection series, rate of yolk deposition, as measured in terms of actually ovulated yolk substance, was increased for at least a time. If her record may be accepted, this is readily evident in the case of hen 2477 (table 3), and to a lesser degree the same is true of hens in which ovulations were forced 4 or 5 times successively, due account being taken of the interval between injections. Since none of the birds were autopsied at the conclusion of tests, it is difficult to know whether the limited data indicate true follicular stimulation or a differential effect

on larger follicles only as a result of increased frequency of the ovulation-inducing stimulus. This phase of the work calls for further study.

If in fact the possibility of obtaining sequences of greater length than 4 or 5 added ovulations was limited by failure of the follicles to increase their rate of growth to match the increased rate of ovulation, it should be of interest to increase rate of follicular growth by administering follicle-stimulating hormone concurrently with injections to force ovulation at appropriate intervals. Pregnant mares' serum is relatively highly effective in stimulating follicular growth in low producing hens (Fraps, 1940), and although the stimulated follicles do not normally ovulate, they respond readily to lutein'zing preparations (Fraps and Riley, 1942). An appropriate combination of these two agencies might make possible considerably longer consecutive ovulation sequences than are recorded in this paper, and possibly also the interval between successive ovulations might be reduced even below 24 hours.

SUMMARY

Ovulations were induced in hens of low to moderate production at about the time of lay of the terminal egg of a 2-egg clutch, and at succeeding intervals of approximately 24, 26 or 28 hours until the ovary failed to respond. Air-dried anterior pituitary tissue of mature male chickens and progesterone were used to force ovulation.

Excluding 3 birds recently placed in cages, the following additions were made to the expected 2-egg clutch of each of 50 hens: in 6 hens, 5 eggs; in 7 hens, 4 eggs; in 11 hens, 3 eggs; in 10 hens, 2 eggs; in 13 hens, 1 egg; in 3 hens, none. In at least the longer sequences, the decrease in weight of yolks was more rapid than in normal clutches of equal length, suggesting that rate of follicular growth limited the number of ovulations possible in these hens. Of 60 hens serving as controls, only 4 laid clutches of more than the anticipated 2 eggs; these 4 laid 3-egg clutches.

Delays of 1 to 4 days beyond the expected time of lay were observed in 4 hens receiving relatively high dosages of progesterone.

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ENDOCRINE INFLUENCES ON IMPLANTATION AND DECIDUOMA FORMATION[1,2]

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IT HAS been well established (Rothchild and Meyer, 1942) that deciduoma formation is dependent on adequate amounts of progestational hormone and that estrogenic substances, even in minute amounts, will inhibit the reaction if present just prior to the eliciting stimulus. It is also well known (Allen, 1931; Selye and McKeown, 1935; Peckham and Greene, 1947) that the deciduomal reaction in the rat can be elicited through the 7th day of pregnancy but not thereafter. Two explanations have been offered for the loss of endometrial reactivity after the 7th day: (a) Reduction in progesterone secretion below levels necessary for reactivity. (b) Appearance of inhibitory agents.

The first of these possibilities is eliminated by the finding (Peckham and Greene, 1949) that reactivity of the endometrium cannot be restored by the administration of even large amounts of progesterone. In order to investigate the possible role of inhibitory factors, the effect of removal of various structures which might be sources of inhibitors was determined. In the course of these experiments it was possible to confirm the previously unconfirmed report (Rothchild and Meyer, 1940) that implantation will occur and pregnancy can be maintained in the castrate rat treated with progesterone alone.

METHODS AND RESULTS

The remaining tube and ovary of 8 unilaterally pregnant rats (175 to 275 gm.) were removed on the 5th day of pregnancy[3] (after the ova had entered the uterus, but before implantation) and 2.0 mg. of progesterone in oil were administered subcutaneously daily through the 11th day. At laparotomy on the 9th day all animals were found to have implanted normally (2 to 8 sites per rat, average of 5.5). At this time the non-pregnant uterus of each rat was stimulated at 4 points by passing a suture transversely through the uterus and leaving it loosely tied *in situ*. At sacrifice on the 12th day 2 of 8 rats were found to have deciduomata indicating that the inhibitory

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[2] Progesterone used in these experiments furnished through the courtesy of Ciba Pharmaceutical Products Inc. and Schering Corp.

[3] The day that sperm were observed in the vaginal smears was considered day one of pregnancy.

TABLE 1. DECIDUOMATA AND IMPLANTATIONS IN UNILATERALLY PREGNANT RATS TREATED WITH 2.0 MG. PROGESTERONE DAILY

Rat no.	No. of sites on 9th day	No. of sites on 12th day*	No. of deciduomata formed	Microscopic appearance on the 12th day
* 1	7	5	3	1 normal 3 resorbing 1 no fetal tissue seen†
2	6	6	0	4 normal 2 resorbing
3	6	6	0	2 normal 2 resorbing 2 no fetal tissue seen†
5	5	5	0	3 normal 2 resorbing
7	5	5	0	1 normal 4 resorbing
8	7	2	0	2 resorbing
10	8	4	1	4 no fetal tissue seen, peritonitis†
11	2	2	0	2 normal

* Sites obviously in poor condition not included or sectioned.
† Single sections, fetal tissue possibly present elsewhere in site.

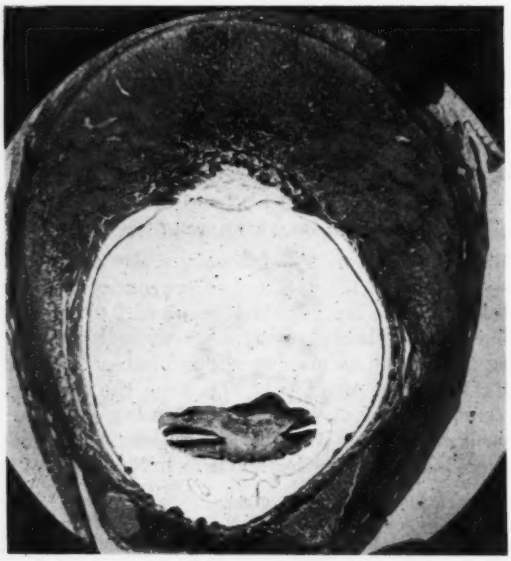

FIG. 1. A well developed, 12th day implantation site from a castrate, progesterone treated rat. No deciduomata developed in the non-pregnant uterus of this rat.

influences had been reduced although not abolished by removal of the ovary. Healthy embryos and placentas, comparable to normal pregnancies of comparable age (see Fig. 1) were found on microscopic examination in 6 of the 8 rats. In one of the remaining two, recent fetal death had occurred judging from the necrosis and hemorrhage. No deciduomata formed in this animal. In the other no fetal tissue was observed in any of the sites examined. This animal as well as one with one normal embryo developed deciduomata (see Table 1).

When the experiment was repeated except that the remaining tube and ovary were removed on the 4th day (thus also removing the products of conception) deciduomata developed in all 10 rats indicating that inhibitory influences originated in part from the products of conception.

That estrogen, which is formed by the ovaries and possibly by the fetal trophoblast, is sufficiently potent to suppress completely the deciduomal response was shown in a third group of 6 rats treated exactly as in the previous experiment, except that 1.0 gamma of estrone was added to the daily dose of 2.0 mg. of progesterone. None of these animals, in contrast to the previous series, developed deciduomata.

DISCUSSION

It has previously been demonstrated (Peckham and Greene, 1947) that removal of the products of conception without disturbing the

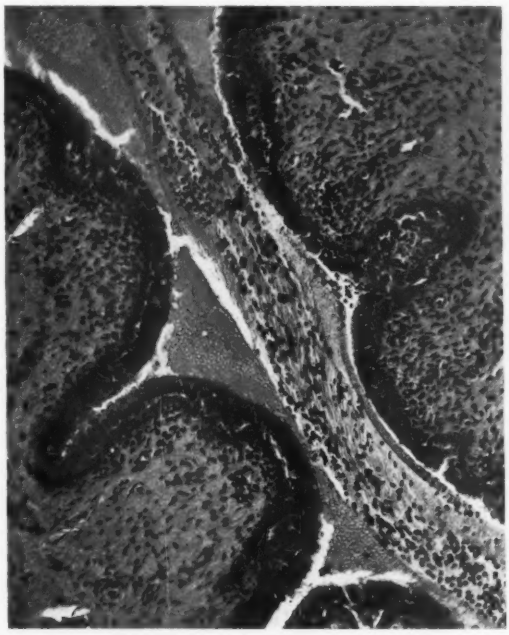

FIG. 2. Vagina of a castrate pregnant rat treated with 2.0 mg. progesterone daily for 7 days after castration. This section is from the vagina of the rat from which the pregnancy in Figure 1 was obtained.

ovaries on the 7th day of pregnancy does not restore the deciduoma reaction. This finding and the present results lead to the conclusion that the loss of endometrial sensitivity after the 7th day of pregnancy in the rat is due to the presence of an inhibitor which originates both in the ovaries and fetal trophoblast and is presumably estrogen. If the inhibitor is estrogen, the amount produced by the developing embryo

FIG. 3. Vagina of a castrate pregnant rat treated with 2.0 mg. progesterone plus 1.0 gamma estrone daily for 8 days.

must be very small as the vaginas from the rats with the embryos *in situ* and those with the embryos removed were found to be identical on microscopic examination. The vaginas of these animals showed the low degree of mucification elicited by progesterone treatment in the castrate (see Fig. 2). The inhibitor was able to prevent the formation of deciduomata in 6 of the 8 rats yet was not able to produce a demonstrable change in the vaginal epithelium. In the remaining rats treated with 1.0 gamma estrone daily in addition to the progesterone the vaginal epithelium showed a high degree of mucification similar to that seen in pregnant rats retaining their ovaries about the 12th day of pregnancy (see Fig. 3). Obviously 1.0 gamma estrone daily is considerably more "estrogen" than is produced by the fetal tissues.

SUMMARY

Failure of the rat endometrium to form deciduomata in response to a mechanical stimulus after the 7th day of pregnancy is due to the presence of an inhibitor, probably estrogen, which originates both in the maternal ovaries and in the fetal trophoblast, since:

a) Removal of both ovaries partially restored the deciduomal response (8 rats),
b) Removal both of ovaries and products of conception completely restored it (10 rats),
c) The addition of the daily administration of one gamma of estrogen completely suppressed the reaction again (6 rats).

The claim of Rothchild and Meyer (1940) that implantation occurs and pregnancy is maintained in the absence of estrogen in the castrate rat treated with 2.0 mg. progesterone daily is confirmed.

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THE EFFECTS OF GRADED DOSAGES OF ESTROGEN UPON PITUITARY CYTOLOGY AND FUNCTION[1]

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DOES ESTROGEN stimulate or suppress the gonadotrophic activity of the pituitary gland? As early as 1930, Burch and Cunningham reported that injections of the estrus hormone (from human placenta) into castrated female rats produced an increase in the amount of gonad-stimulating hormone of the hypophysis as determined by implantation of this gland into immature female mice. However, Meyer, Leonard, Hisaw and Martin (1930, 1932), using more potent estrogenic preparations and longer periods of injection, demonstrated that estrin decreases the amount of gonad-stimulating complex in the hypophyses of castrated male and female rats when compared with castrated, uninjected animals. Wolfe (1931) suggested that a large amount of estrin may inhibit the production of the luteinizing hormone and that a small amount might stimulate its production. This concept was supported by Meyer, Biddulph and Finerty (1946) who found increased gonadotrophic content of the pituitary glands and increased percentage of acidophiles in ovariectomized rats following injection of small doses of estradiol for 10 days. Baker and Everett (1944), injecting small quantities of diethylstilbestrol for 4 or 7 days, found increased mitotic activity, hypertrophy of the Golgi apparatus and increase in number of acidophiles accompanying degranulation and reduction in number of basophiles. Most recent work correlates cells of the acidophilic type with the luteinizing component of the pituitary gonadotrophic complex (see Dawson, 1946).

The present experiments were planned to compare the effects of small, moderate and large amounts of estrogen injected for a constant period of 10 days into ovariectomized immature rats. Quantitative cell counts were used to determine the effects on pituitary cell types and gonadotrophic hormone content was ascertained by injection of

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homogenized pituitary tissues into hypophysectomized immature female rats and noting the response of the ovaries.

PROCEDURE

These experiments include observations on the effects of varied amounts of estrogen on 153 ovariectomized rats. For control purposes, 67 untreated ovariectomized and 68 untreated intact female rats were used. Quantitative cell counts of the pituitary glands were made on 66 and gonadotrophic assays on the pituitary glands of 126 of the above animals. Forty-five hypophysectomized immature female rats were used as assay recipients. All of the animals were obtained directly from the Holtzman Rat Company.

All rats, except the unoperated controls, were ovariectomized on the 31st day of life. Subcutaneous injections were started on that day and made daily for a total of 10 days. Animals of all groups were autopsied at 41 days of age.

The estrogen used in this study was dienestrol (4:4′-di-hydroxy-γ-δ diphenyl-β-δ-hexadiene)[3] made up as a suspension in 0.9% saline solution containing 0.5% chlorobutanol. The stock solution contained 1.0 mg. per ml. and dilutions were made so that each daily dose was contained in 0.1 ml. of solution.

Seven groups of rats were studied cytologically: untreated; ovariectomized; ovariectomized and injected daily with .025 μg. dienestrol; ovariectomized and injected daily with .10 μg. dienestrol; ovariectomized and injected daily with .50 μg. dienestrol; ovariectomized and injected daily with 2.0 μg. dienestrol; and ovariectomized and injected daily with 5.0 μg. dienestrol. For cytological study the pituitary glands were fixed immediately in Zenker-formol solution, sectioned at 4 microns and stained with the modified azocarmine method described by Briseno-Castrejon and Finerty (1949). The average percentage of each cell type was estimated by differentially counting all the cells in every fifth field of a horizontal section through the middle of each gland. In previous studies (Finerty, Meyer, and Marvin, 1944; Meyer, Biddulph and Finerty, 1946) it has been the practice to differentially count the cells in three horizontal sections at equidistant levels in each gland. Careful study of these counts reveals that there is no significant change in the resulting percentages if only a single central section is counted, so in this project the latter method is utilized which makes it practicable to observe more animals.

The pituitary glands of four groups of rats were assayed for gonadotrophic content: untreated; ovariectomized; ovariectomized injected daily with a low dose of estrogen (.10 μg. dienestrol suspension); and ovariectomized injected daily with a high dose of estrogen (5.0 μg. dienestrol suspension).

RESULTS

The effects of ovariectomy and of injection of graded doses of dienestrol upon body and organ weights of immature female rats are summarized in Table 1.

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[3] We are indebted to Dr. E. R. Neary of the White Laboratories, Inc. for the dienestrol used in these experiments.

TABLE 1. THE EFFECT OF OVARIECTOMY AND ESTROGEN TREATMENT ON THE AVERAGE WEIGHT OF VARIOUS ORGANS OF IMMATURE FEMALE RATS

Treatment	No. of rats	Body weight (gm.)	Ovarian weight (mg.)	Uterine weight (mg.)	Adrenal weight (mg.)	Thymus weight (mg.)	Pituitary weight (mg.)
Untreated	68	112. ±1.2*	28.9 ±1.2	97.3 ±9.0	28.5 ±0.5	370. ±8.0	5.1 ±0.1
Ovariectomized (10 days)	67	100. ±1.8		30.3 ±0.9	28.7 ±0.4	342. ±8.5	5.1 ±0.1
Ovariectomized plus .025 μg. dienestrol per day for 10 days	10	122. ±3.5		47.7 ±5.4	32.0 ±1.5	453. ±27.	—
Ovariectomized plus .10 μg. dienestrol per day for 10 days	69	103. ±1.3		62.9 ±1.0	29.5 ±0.4	354. ±8.7	5.5 ±0.1
Ovariectomized plus .50 μg. dienestrol per day for 10 days	9	108. ±5.3		124.0 ±7.7	30.6 ±1.0	358. ±48.	—
Ovariectomized plus 2.0 μg. dienestrol per day for 10 days	10	102. ±2.5		152.0 ±10.1	27.8 ±1.1	269. ±14.	—
Ovariectomized plus 5.0 μg. dienestrol per day for 10 days	65	95. ±1.2		178.0 ±3.7	28.7 ±0.4	249. ±3.9	6.0 ±0.1

* Standard error.

Moderate amounts of dienestrol seem to exert little effect upon growth at this period, as shown by body weights, but the highest dose level (5.0 μg. dienestrol per day for 10 days) has a marked inhibiting effect on body growth. While the rats of the control group were gaining approximately 40 grams during the 10 day experimental period, those rats receiving 5.0 μg. dienestrol gained about 20 grams. This may be due to a specific inhibition of growth hormone production, but it seems more likely that it is a non-specific response to a high level of a toxic substance.

Uterine weights of intact rats at 41 days of age are extremely variable and in this study their range is from 29.0 to 378.0 mg. with a mean of 97.3 mg. Such variation is a reflection of the rapidity with which the female sexual organs are developing at this age, so that slight differences in sensitivity of the end organs or slight differences in onset of increased estrogen secretion are greatly magnified. However, following ovariectomy variation in uterine weight is much reduced, and this condition is maintained when groups of ovariectomized rats are injected with equal amounts of estrogen (compare the standard errors of groups which include 60 rats or more). For example, the range of weights of the uteri of uninjected ovariectomized rats is 19.0 to 49.2 mg. with a mean of 30.3 mg.; the range for the highest

estrogen injected group is 119.0 to 262.0 mg. with a mean of 178.0 mg.

Mean weights of the adrenal glands are remarkably constant in these seven groups but if the relation of the adrenal weight to body weight is considered there seems to be a tendency toward adrenal hypertrophy in the highest estrogen group. The thymus weight shows progressive decrease with increase in estrogen concentration.

During the 10 day period of experiment there was no change in weight of the hypophysis due to ovariectomy alone. However, injection of a low dose of estrogen (.10 μg. dienestrol per day) increased the mean weight of the gland 8% (.4 mg.); and injection of a high dose of estrogen increased the mean weight by 18% (.9 mg.). Since the differentiation between the mean pituitary weights of control rats and low estrogen-treated rats is approximately three times the standard error of the difference, the increase in weight is statistically significant. The increase in weight of the high estrogen-treated rats is statistically highly significant. These small, but significant, increases in weight of the hypophysis with relatively short periods of estrogen treatment substantiate the results of Baker and Everett (1944).

Pituitary cell type analysis: The results of the quantitative cell

TABLE 2. THE EFFECT OF OVARIECTOMY AND ESTROGEN TREATMENT ON CELL TYPES AND GONADOTROPHIC CONTENT OF THE PITUITARY GLANDS OF IMMATURE FEMALE RATS

Treatment	Pituitary cell type analysis				Pituitary assay data	
	No. of rats	% Acid.	% Baso.	% Chrom.	No. of rats	Ovarian weight (mg.)
Untreated	10	38.9 ±1.2*	6.2 ±0.2	54.9 ±1.2	4	13.2 ±0.4
Ovariectomized (10 days)	10	39.1 ±0.9	15.9 ±0.5	45.0 ±1.1	6	72.9 ±9.6
Ovariectomized plus .025 μg. dienestrol per day for 10 days	10	44.4 ±1.6	10.1 ±0.5	45.5 ±1.7	—	—
Ovariectomized plus .10 μg. dienestrol per day for 10 days	12	47.4 ±1.5	5.2 ±1.0	47.4 ±1.2	8	74.8 ±11.4
Ovariectomized plus .50 μg. dienestrol per day for 10 days	9	41.7 ±2.7	3.1 ±0.7	55.2 ±3.2	—	—
Ovariectomized plus 2.0 μg. dienestrol per day for 10 days	10	35.8 ±2.0	3.1 ±0.6	61.1 ±3.2	—	—
Ovariectomized plus 5.0 μg. dienestrol per day for 10 days	5	31.2 ±1.0	0.9 ±0.3	67.9 ±1.0	11	28.6 ±2.2
Uninjected assay controls					16	9.9 ±0.4

* Standard error.

counts are summarized in Table 2. The basophilic cells of the pituitary gland show a remarkably consistent response to the level of injected estrogen. Ovariectomy, alone, results in a change in percentage of basophiles, from 6.2 to 15.9, which is a well-known response to reduction in ovarian hormones. With each increase in amount of estrogen injected there is a progressive decrease in percentage of basophiles, so that with injection of .10 μg. dienestrol a day the castration picture has reverted to that of the intact state, and with the injection of 5.0 μg. a day the basophiles make up less than 1% of all the cells. Percentage of cells alone does not delineate the true change in amount of basophilic material, however. The result of castration is not only an increase in percentage of basophiles, but a striking increase in the size of individual cells. The short ten-day period of castration does not show the "signet-ring" cells characteristic of more prolonged absence of gonadal hormones, but many of the basophiles are much enlarged and exhibit prominent negative Golgi images. These appear to be highly active cells, and the high pituitary gonadotrophic content on assay, as well as the high gonad-stimulating power of castrates in parabiosis tend to strengthen this concept.

Ovariectomy has little or no effect on percentage of acidophiles over the short period of 10 days, although there is evidence to support the thesis that long periods of castration result in a slight decrease (Severinghaus, 1933). Daily injection of estrogen starting at the day of ovariectomy for the ten day period has diverse effects upon acidophiles, depending upon the dosage. The two lower doses of dienestrol caused statistically significant increases in the percentage of acidophiles with the greatest increase occurring at a level of .10 μg. per day. The lowest amount used (.025 μg./day), which reduced the basophilic percentage to 10.1 per cent and stimulated uterine growth indicating a partial estrogen replacement, induced an increase in percentage of acidophiles to 44.4 per cent, a statistically significant increase. At the .10 μg. dosage, the percentage of acidophiles reached 47.4 per cent, and the basophiles were returned to a normal level. Complete replacement of the normal estrogen check on pituitary cell types was attained at .50 μg. dienestrol per day. Uterine weight in the normal range was also attained between the latter two dosages. Increasing the amount of dienestrol injection above this level resulted in a progressive decrease in percentage of acidophiles so that with the 5.0 μg. dose level there was only 31.2 per cent acidophilic cells.

Pituitary gonadotrophic assay: Results of gonadotrophic assays of the pituitary glands are tabulated in Table 2. Female rats hypophysectomized at the age of 25 days and autopsied on the morning of the 31st day of life served as the controls and exhibited ovaries weighing 9.9 mg. Injection of homogenized fresh pituitary gland tissue (total of 15 mg.) injected into similarly prepared recipients resulted in 13.2 mg. ovaries when the donors were untreated 41 day old female rats.

Ovariectomy of the donors for a 10 day period increased pituitary gonadotrophic content as shown by 72.9 mg. ovaries of the recipients. Injection of a low dose of dienestrol during the 10 days of ovariectomy of the donors resulted in 74.8 mg. ovaries; whereas, injection of a high dose of dienestrol resulted in 28.6 mg. ovaries. Since gonadotrophic assay is a reflection of the combined effect of follicle stimulating and luteinizing fractions of the gonadotrophic complex it is difficult to draw rigid conclusions concerning these values. However, it is obvious that ovariectomy increases the gonadotrophic content of the hypophysis; that a low amount of estrogen does not change this value (even though the basophile count is lowered); and that a high estrogen level prevents the attainment of a high gonadotrophic content.

DISCUSSION

These data support and extend previous observations in which the action of various estrogens has been found to be stimulatory to the gonadotrophic function of the hypophysis, as well as those in which estrogen has been considered inhibitory. Evidence was presented by Baker and Everett (1944) and by Meyer, Biddulph and Finerty (1946) that the effect of an estrogen may be dependent upon the amount administered and upon the duration of injection. The present series of experiments utilized animals of the same age and physiological state, treated for a constant period of ten days, with dosage of estrogen as the only variable. It can be seen that when minute quantities of estrogen are supplied to an ovariectomized rat there is an increase in percentage of acidophilic cells, a drop in percentage of basophiles and a negligible increase in gonadotrophic potency. This effect, in response to dienestrol, substantiates the results of Baker and Everett (1944) who used diethylstilbestrol, and those of Meyer, Biddulph and Finerty (1946), who used estradiol. The change in cell types is not of the magnitude observed by the former workers, nor is the increased potency as striking as that of the latter group, but the changes are in the same direction. Increasing the amount of estrogen administered progressively decreases the percentage of basophiles. However, response of the acidophiles to increasing amounts of estrogen is at first an increase in percentage, reaching a peak at a dosage of .10 μg. dienestrol per day, followed by progressive decreases in percentage with increasing doses.

Assay data support this differential effect in that castration alone results in a marked increase in gonadotrophic content of the hypophysis, primarily follicle stimulating in nature, and presumably a reflection of the marked increase in number and development of the basophile cells. Following injection of a low dose of estrogen the gonadotrophic content of the pituitary gland is not significantly different from the ovariectomized uninjected group even though the percentage of basophiles has been markedly reduced. The change in

gonadotrophic potency in this experiment is negligible, but the fact that there is no decrease suggests that the gonadotrophic content is maintained as an expression of the increased acidophile concentration. Injection of a high dose of estrogen prevents the increase in gonadotrophic content which normally follows ovariectomy.

These assay data fit in well with the work of Lauson, Heller and Sevringhaus (1938), who found that estrogen administered in moderate doses did not prevent the post-castration rise in gonadotrophins in rats. Similar observations were the failure of physiological amounts of estrogen to lower post-menopausal rise of urinary gonadotrophins in women (Heller, Heller and Sevringhaus, 1942); or to suppress urinary gonadotrophins in castrated women (Heller, Chandler and Myers, 1944). These, and other observations, led Jungck, Heller and Nelson (1947) to conclude that "physiological amounts of estrogen exert very little inhibitory action upon the pituitary," whereas "large, unphysiological doses of estrogen are potent inhibitors of pituitary gonadotrophic potency." The observations presented in this paper substantiate these conclusions, but suggest that the mechanism of the failure of inhibition of pituitary gonadotrophic secretion is a simultaneous suppression of basophilic FSH secretion and stimulation of acidophilic LH secretion. The end result is, therefore, a maintenance of the high gonadotrophic content of the untreated ovariectomized state.

By these simultaneous studies of cell types and gonadotrophic content of the hypophyses of ovariectomized rats using controlled dosages of synthetic estrogen for a constant period of injection it now seems possible to harmonize many of the seemingly contradictory conclusions reached by previous investigators. Those workers who attributed a pituitary stimulatory action to estrogen uniformly used low concentrations or short periods of injection, whereas those who concluded that estrogens inhibit gonadotrophic secretion gave high doses over longer periods. Kraul (1931), who attributed an increase in luteinizing hormone content of the hypophysis to estrogen treatment, implanted pituitary glands of rabbits which had received daily injections of an extract of human placenta for six days into immature mice and obtained marked luteinization of the recipient's ovaries. Burch and Cunningham (1930) injected a commercial preparation of placental extract (5–25 r.u.) into ovariectomized rats for six days and on assay obtained heavier ovaries than when implants of pituitary glands from uninjected castrate female rats were used. On the other hand, those who attribute an inhibitory effect of estrogen on gonadotrophic secretion used higher doses or prolonged periods of injection. Meyer *et al.* (1932), studying the influence of "long period injections of the oestrous hormone" found a decisive decrease in gonad-stimulating power of the hypophyses of estrogen-injected castrated rats when compared with the hypophyses of non-injected castrated animals.

Cytological studies (Severinghaus, 1937; Wolfe, 1949) have led

to the conclusion that the degranulation of chromophiles and decrease in gonad-stimulating hormone content which results from prolonged estrogen treatment are a measure of high secretory activity rather than an indication of suppressed function. Implantation, or injection, of pituitary suspensions from rats which had been injected with estrogen has led other investigators to conclude that estrogen inhibits production of gonadotrophic substances. It is true that assay methods, as argued by supporters of the former theory, measure hormone content only and give no indication of the relative activity of the gland; but if estrogen stimulates high secretory activity it is difficult to explain the results of estrogen injections in parabiotic rats. Estradiol injection into castrated partners in parabiosis prevents hypersecretion of pituitary gonadotrophin, so that normal adult partners have regular cycles (Meyer and Biddulph, 1941), or so that immature partners undergo no ovarian hypertrophy (Meyer, Biddulph and Finerty, 1946). Hellbaum and Greep (1946) have put forth another explanation, namely that estrogen liberates the hypophyseal luteinizing factor, which in the absence of estrogen remains stored within the pituitary gland.

The data presented in this paper show that there is no increase in percentage of basophiles following low doses of estrogen, which agrees with the results of Baker and Everett (1944). No substantiation is found in these results for the statement of Severinghaus (1937) that "the early response to light or moderate dosage is a stimulation of the basophiles." The former workers assert that the increased acidophilia occurs at a level too low to significantly affect the basophiles. Nevertheless we find that even the lowest amount of dienestrol (.025 μg./day) has marked effect on lowering the increased basophile percentage of castration. This disagreement might easily be due to a difference in sensitivity between the pituitary gland of a castrate and that of an intact immature rat since the experiments of Baker and Everett were on intact rats. Foster (1942) observed that a state of pituitary "secretory exhaustion" was produced in immature rats by implantation of pellets of dienestrol or stilbestrol; and that the acidophiles showed a greater resistance to the effects of estrogens than did the basophiles.

On the basis of the data presented the following conclusions are suggested:

1. Pituitary gonadotrophic activity is influenced by the amount of circulating estrogen, which at low concentrations stimulates acidophilic cells and luteinizing hormone secretion: inhibits basophilic cells and follicle stimulating hormone secretion; and at high concentrations suppresses all gonadotrophic function.

2. Estrogen at any concentration reduces the number and activity of pituitary basophile cells, in direct proportion to the concentration of estrogen.

3. Estrogen in low concentrations increases the percentage of pituitary acidophiles and in high concentrations reduces their number.

4. Dienestrol exerts effects on the structure and function of the hypophysis similar to those of estradiol and diethylstilbestrol.

SUMMARY

Histological studies and gonadotrophic assays were made of the pituitary glands of immature ovariectomized rats which had been injected with graded amounts of dienestrol (.025 μg. to 5.0 μg./day) for a period of ten days.

The high pituitary gonadotrophic content of castration was not changed significantly by low estrogen injection (.10 μg. dienestrol/day) but was markedly reduced by high estrogen (5.0 μg. dienestrol/day). The percentage of basophile cells was decreased in direct proportion to the concentration of estrogen. Acidophilic cells were increased in percentage by the lower doses reaching a peak of 47.4 per cent at .10 μg. dienestrol per day, and decreased by the highest doses to 31.2 per cent, compared to 39.1 per cent in the uninjected ovariectomized controls.

Dienestrol exerts effects upon the structure and function of the hypophysis similar to those of estradiol and diethylstilbestrol.

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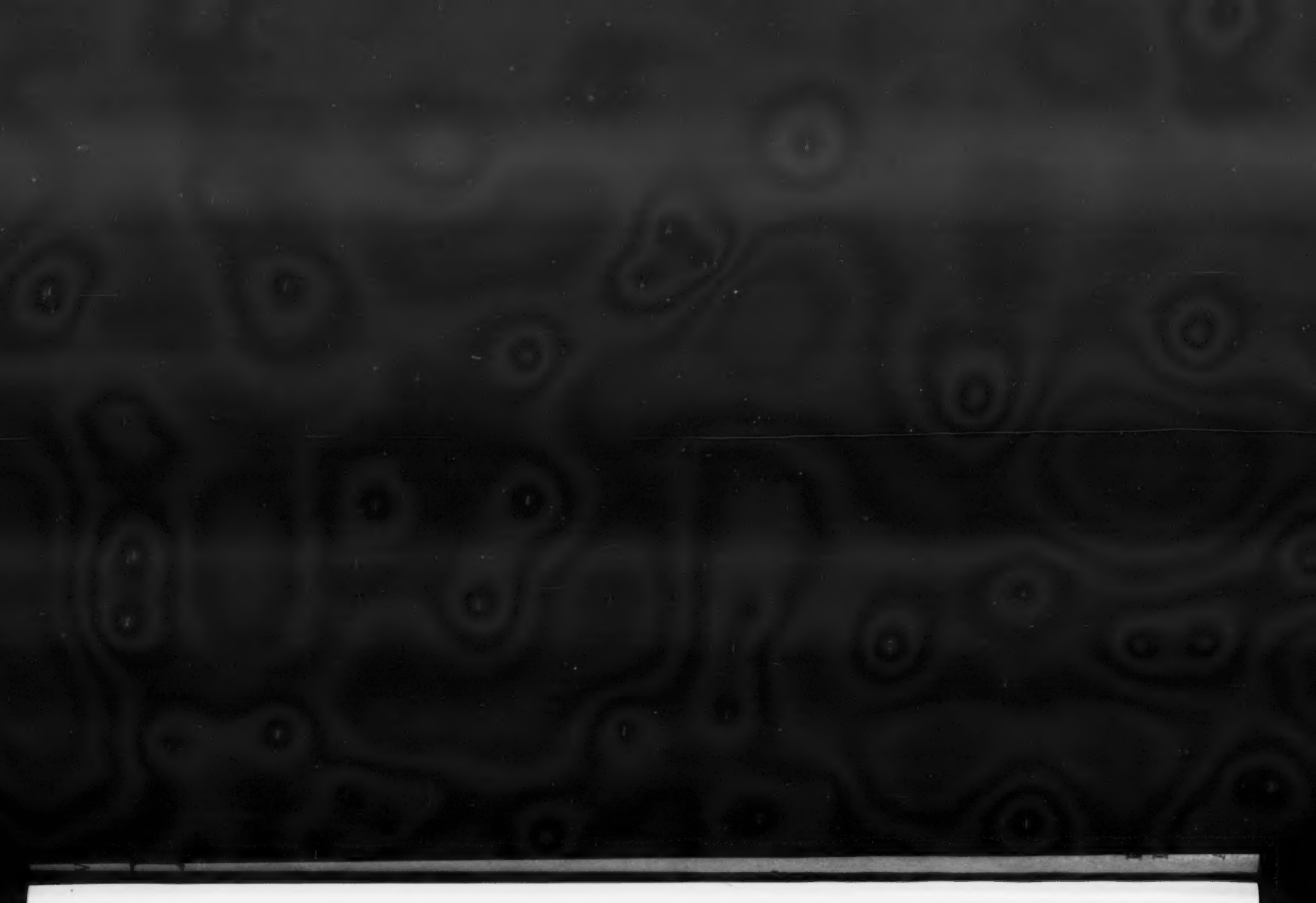
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